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CREATININE FORMATION FROM CREATINE BY YEAST

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It has been generally recognized that creatinine in urine is formed spontaneously from creatine in the animal body (*1*). On the other hand, some authors pointed out that the amount of creatinine in urine is closely related to the carbohydrate metabolism in the animal body. For example, in the case of diabetes, excretion of creatine in urine occurs together with the decline of carbohydrate metabolism (*2*). The creatinuria owing to the starvation can be recovered by the administration of carbohydrate, but neither protein nor fat proves to be effective (*3*).

The problem of the correlation between carbohydrate metabolism and the creatinine formation from creatine in yeast cells was studied by T. Soda and K. Shimokata, who showed that the conversion in question occurred only when sugars were fermented by yeast cells (*4*).

The authors investigated this problem once again and more precisely. It was found that the creatinine formation was very small under aerobic condition compared with that under anaerobic condition when baker's yeast was used. Under aerobic condition, the fermentation was suppressed due to the Pasteur effect, and the creatinine formation may have been reduced. As the creatinine formation was completely inhibited by NaN_3, phosphorylation may as well participate in this process.

EXPERIMENTAL METHODS AND EXPERIMENTAL RESULTS

In this experiment, baker's press yeast (Oriental Yeast Co. Ltd.) and Fleischmann's dry yeast were used. Before the experiment, the yeast cells were washed twice with distilled water by centrifugation.

The reaction mixture contained 5 per cent glucose, 0.1 per cent creatine, yeast cells (1 g. in wet weight of baker's yeast or 0.2 g. of Fleischmann's dry yeast) and several reagents (total volume 10 ml.), and was incubated for a day at 30°. During the fermentation as well as respiration, the medium became gradually acidic.

So it was necessary to add 10^{-1} *M* NaOH at times in order to keep the pH over 5.0*, because creatine is converted spontaneously to creatinine with considerable rate in acidic medium.

The amount of creatinine formed was estimated by Folin's method (*5*) using Jaffe's reaction by photometiric method. Jaffe's reaction is not specific to creatinine: acetone and acetoaldehyde *etc.* react with sodium picrate. Some fermentation products can show the same reaction, so this fraction must be corrected in order to estimate the true amount of creatinine. The amount of creatinine spontaneously converted from creatine must also be subtracted as blank (Table I).

We adopted the paper chromatographic method in order to identify creatinine formed in the reaction mixture. After incubation for a day, the reaction mixture was extended using the mixture of phenol, acetic acid and water (4:1:2) and, after dried in room temperature, the papers was sprayed with a saturated solution of sodium picrate. The Rf value of the spot was 0.63, and no other spot could be recognized. The Rf value of creatinine was 0.62 under the same condition, and that of creatine was 0.52 which was recogized after its conversion to creatinine by heating. So it could be assured that creatinine was formed from creatine in the reaction mixture.

Table II and Table III show the creatinine formation under various conditions. Several important facts have emerged from these results.

Correlation between Creatinine Formation and Fermentation—Experiments with baker's yeast showed that the formation of creatinine occurs in a larger measure under anaerobic condition than under aerobic condition. Such a defference was not observed with Fleischmann's yeast. Baker's yeast produces twice as much CO_2 under anaerobic condition as under aerobic condition ($Q_{CO_2}^{aer.}/Q_{CO_2}^{anaer.} \fallingdotseq 0.5$) (Table V), whereas Fleischmann's yeast, shows no difference in CO_2 production under aerobic and supressed anaerobic conditions, indicating the lack of the Pasteur effect ($Q_{CO_2}^{aer.}/Q_{CO_2}^{anaer.} \fallingdotseq 1.0$). We have also seen that the respiration caused no decrease of creatinine added. When alcohol was respired in the reaction mixture in place of glucose, creatinine was not formed.

These facts indicate that the creatinine formation is coupled with the fermentation of glucose by yeast cells.

Correlation between Creatinine Formation and Phosphorylation—It is well known that NaN_3 has no effect on the fermentation, but inhibits the phosphorylation completely. We have seen that the creatinine formation was inhibited by NaN_3. On the other hand, 2,4-dinitrophenol and

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* In the case of baker's yeast, about 1.5 ml. of 10 *M* NaOH was required, and in the case of Fleischmann's yeast about 0.5 ml. If buffer solutions, such as succinate and glycine buffers, were used, considerably higher amount of alkali must have been added in order to keep the medium over pH 5.0. Therefore, we did not use the buffer solution.

TABLE I

Creatinine Formation during the Fermentation

Reaction mixture	Final pH	Apparent amount of creatinine produced	True amount of creatinine produced
Complete	5.6	280γ	220γ
No glucose	5.4	15	
No creatine	5.6	45	

The complete reaction mixture contained 5% glucose, 0.1% creatine, 10^{-3} M KCN and 1.0 g. (wet weight*) of baker's yeast; total volume was 10 ml. Before and after the incubation, a portion of the reaction mixture was centrifuged, and the amount of creatinine was estimated with the supernantant solution.

* About 0.3 g. in dry weight.

TABLE II

Creatinine Formation by Baker's Yeast under Various Conditions

Yeast condition	KCN (10^{-3} M)	Reagent added	Creatinine produced	Expt. No.
Fresh yeast, inorganic phosphate and acid labile phosphate was 5γ/mg. dry yeast	Added		360 ±10γ	I
			80	
	Added		340	II
		Anaerobic condition*	350	
	Added		320	III
		2.5×10^{-4} M NaN_3	Nil	
		Alcohol**	Nil	
			90	
Phosphate starved yeast, inorganic phosphate and acid labile phosphate was 1.7γ/mg. dry yeast	Added	2×10^{-2} M Phosphate	270	IV
	Added		120	
	Added	2×10^{-2} M Phosphate plus 5×10^{-5} M DNP	290	
	Added	2×10^{-2} M Phosphate plus 10^{-3} M Be^{++}	240	
	Added		300	V
	Added	2.5×10^{-4} M NaN_3	Nil	

The reaction mixture contained 5% glucose, 0.1% creatine, 1.0 g. (wet weight) of the baker's yeast and the reagent indicated; total volume 10 ml.

* Using Thunberg tubes, the reaction mixture was incubated *in vacuo*: or with gas space replaced with CO_2.

** In this case glucose was replaced by alcohol (0.25 ml.).

TABLE III

Creatinine Formation by Fleischmann's Yeast under Various Conditions

Yeast condition	KCN (10^{-3} M)	Reagent added	Creatinine produced	Expt. No.
Fresh yeast, inorganic phosphate and acid labile phosphate was 2.2γ/mg. dry yeast	Added		250 $\pm 10^{\gamma}$	I
			230	
		2.5×10^{-4} M NaN_3	Nil.	
	Added		220	II
			280	
			120	III
		Alcohol*	Nil.	
After phosphate starvation procedure, inorganic phosphate and acid labile phosphate was 2γ/mg. dry yeast	Added		170	IV
	Added	2×10^{-2} M Phosphate	180	
	Added	2×10^{-2} M Phosphate plus 10^{-4} M DNP	180	
		2×10^{-2} M plus 10^{-3} M Be^{++}	100	

The reaction mixture contained 5% glucose, 0.1% creatine, 0.2 g. of Fleischmann's dry yeast and the reagent indicated; total volume 10 ml.

* In this case glucose was replaced by alcohole (0.25 ml.).

TABLE IV

Relation between the Amount of Creatinine Produced and the Concentration of Glucose and Creatine Added

Expt. No.	Creatine added	Creatinine produced
I	0.001 *per cent*	0^{γ}
	0.005	10
	0.01	30
	0.05	80
	0.01	230
Expt. No.	**Glucose added**	**Creatinine produced**
II	2.5 *per cent*	90^{γ}
	5.0	220
	10.0	390

In the Expt. No. I, the reaction mixture contained 5% glucose, 10^{-3} M KCN, 1.0 g. (wet weight) of baker's yeast and creatine as indicated; total volume 10 ml.

In the Expt. No. II, the reaction mixture contained 0.1% of creatine, 10^{-3} M KCN, 0.2 g. of Fleischmann's yeast and glucose as indicated; total volume 10 ml.

TABLE V

Fermentation and Respiration of Yeast under Various Conditions

Yeast condition	Experimental condition	Q_{CO_2}(33°)
Fresh yeast	Air in gas space	93
	CO_2 in gas space	187
	Air in gas space, 10^{-3} M KCN added	182
	Air in gas space, 2.5×10^{-4} M NaN_3 added	174
	Air in gas space, 10^{-3} M KCN and 0.1 % creatine added	214
Phosphate starved yeast	Air in gas space, 10^{-3} M KCN added	
	with 10^{-2} M phosphate	154
	without phosphate	148
Yeast condition	**Experimental condition**	**Q_{O_2} (33°)**
Fresh yeast	Air in gas space, KOH in center well	108
	Air in gas space, 0.1 % creatine added, KOH in center well	93
	Air in gas space, 10^{-3} M KCN or 2.5×10^{-4} M NaN_3 added, KOH in center well	0

The reaction mixture contained 5×10^{-2} M succinate buffer at pH 5.0, 1% glucose, baker's yeast cells and the reagent indicated; total volume 3.0 ml. The oxygen- uptake and CO_2-output were measured by means of Warburg's manometric apparatus.

Be^{++}, both of which are the inhibitors of the oxidative phosphorylation (*6*), but have no effect upon the anaerobic phosphorylation (*7*), did not show remarkable effect on the creatinine formation.

With baker's yeast deprived of phosphate,* the amount of creatinine formed was found to be small if no phosphate was added to the medium. Fleischmann's yeast cells did not lose their inorganic phosphate and acid labile phosphate; and the presence of phosphate had no effect in this case.

These facts suggest us the participation of phosphorylation on the creatinine formation by yeast.

* Yeast cells were washed several times with distilled water, and suspended in a medium containing 2×10^{-3} M $MgSO_4$, 2×10^{-2} M $(NH_4)_2SO_4$, 5×10^{-3} M KCl, 1 per cent glucose and 2×10^{-2} M succinate buffer at pH 5.0. This suspension was kept at 30° and violently aerated for a day. Then the cells were washed several times with distilled water by centrifugation.

The amount of creatinine formed was nearly proportional to the amount of glucose and creatine present (Table IV).

The degree of the fermentation and respiration of baker's yeast under various conditions are shown in Table V. Creatine slightly accelerated the fermentation and inhibited the respiration. NaN_3 and KCN completely inhibited the respiration but were of no effect upon the fermentation.

SUMMARY

1. It was found that, by baker's yeast and Fleischmann's yeast, creatine in media was converted to creatinine accompanied by the fermentation of glucose, but to far less degree by the respiration.

2. Creatinine was not formed during the respiration of alcohol by yeast.

3. NaN_3 completely inhibited the creatinine formation indicating that the process may have some correlation with the phosphorylation.

4. The amount of creatinine formed was nearly proportional to the amount of glucose and creatine present.

It will be interesting to investigate whether creatinine in animal urine may be formed by the similar process in animal body.

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ANTI-THIAMINE FACTORS OF THE FERN

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After Weswig *et al.* (*1*) had reported on the anti-thiamine factors of the ferns, Haag and Weswig (*2*, *3*), Somogyi (*4*), Evans *et al.* (*5*) and Haag (*6*) successively published their studies of these factors. Various biochemical studies are now being carried out to clarify their entity.

The results of the studies reported by these authors seem to coincide on the following points: (a) The inclusion of the ferns in the feed of animals produces the signs of vitamin B_1-deficiency in these animals, although the diet originally contains an adequate amount of thiamine; (b) It has been demonstrated that the anti-thiamine factors include a component which behaves like an enzyme.

These authors seem, however, to have fairly distinctly different views on the entity of these factors.

Pteris aquilina, *Equisetum arvense* L. and *Osmunda japonica* Thunb. have enjoyed a popularity as edible grasses among the peoples of the Orient, especially of Japan. It is interesting to know that people have believed some notices about these vegetables; for example, that dimness of vision results from eating bracken fern very much in early spring, or that people suffering from rheumatism or neuralgia should not eat the bracken fern. Being seriously interested in the significance of the anti-thiamine factors of the ferns in respect to their relations to the nutrition of the Japanese, we have undertaken a biochemical investigation of these factors and further made a human experiment. It has been established by these studies that there are two anti-thiamine factors of entirely different characters in the ferns; namely, a factor which is a kind of thiaminase, and an unknown thermostable anti-thiamine factor.

This report deals with the results of the biochemical investigation performed by us.

EXPERIMENT

Expt. I. Anti-thiamine Activity of Fern Extract at Different pH: Anti-thiamine activity

of extracts at different pH was determined in raw and boiled extracts.

Raw Fern Extract—A mixture of 1 ml. of thiamine solution and 1 ml. of fern extract was added to a series of buffered solutions each adjusted at different pH, and incubated for 30 minutes at 60°. Remaining thiamine was determined by the thiochrome method (*7*) and inactivated thiamine was calculated from it for each member of the series. The results are shown in Fig. 1.

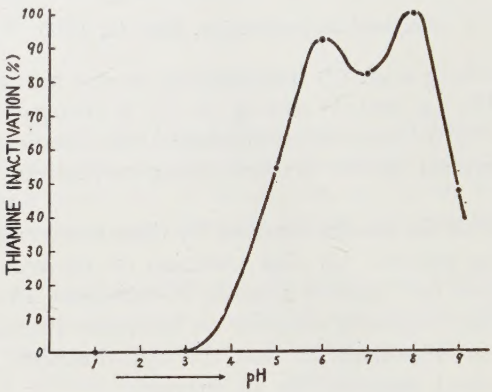

FIG. 1. Thiamine inactivation by fern extract at different pH.

Degree of inactivation was presented in percentage, the inactivation of 15.6 γ at pH 8 being assumed as 100 per cent.

Amount of thiamine used in this experiment was 20 γ. pH 1, HCl-KCl buffer; pH 3 to 6, acetate buffer; pH 7 and 8, phosphate buffer; pH 9, ammoniac buffer. Preparation of fern extract: One part of smashed green leaf of fern was extracted with ten parts of distilled water and centrifuged. The supernatant was used for this experiment.

Boiled Fern Extract—In order to examine the change of anti-thiamine activity by heating, a parallel experiment was performed with 1 ml. of fern extract boiled for 30 minutes at 100°. The results are shown in Fig. 2.

The curve of thiamine inactivation by the fern extract shows two peaks, one at pH 8 and the other at pH 6; the former persists after heating, while the latter disappears. Thus, the presence of two active principles in the fern extract has been established; namely, a thermolabile component active at pH 6 and a thermostable component with the peak at pH 8.

Expt. II. Effect of Oxygen upon Thiamine Inactivation by Fern Extract:

Raw Fern Extract—A parallel experiment with that undertaken in *Expt. I* was performed in nitrogen gas atmosphere each mixture being incubated for 30 minutes at 60°. The results are shown in Fig. 3.

Boiled Fern Extract—Another parallel experiment with fern extract boiled for 30

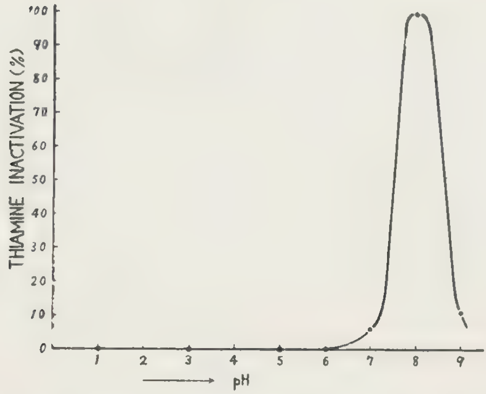

FIG. 2. Thiamine inactivation at different pH by boiled fern extract.

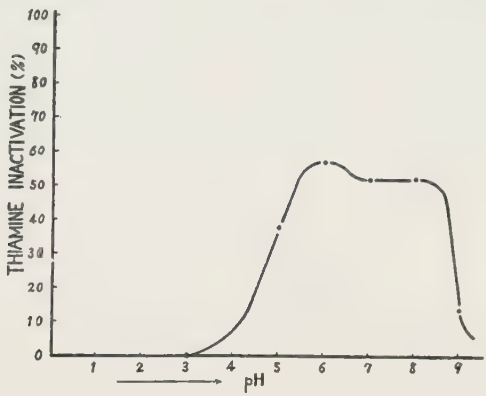

FIG. 3. Thiamine inactivation in nitrogen atomosphere by fern extract.

Amount of thiamine used in this experiment was 50 γ. Degree of inactivation is presented in percentage proportion to 25.5 γ, the amount inactivated at pH 8 by raw fern extract under non application of nitrogen gas.

minutes at 100° as in *Expt. I* was performed in nitrogen gas. The results are shown in Fig. 4.

Anti-thiamine activity curve of fern extract in nitrogen gas atomosphere shows a flat mountain. The peak at pH 8 which characterizes Figs. 1 and 2 disappears, and it is observed that the anti-thiamine activity of the boiled fern extract, having its peak at pH 8, has been strinkingly diminished. Oxygen gas seems to be necessary for thiamine inactivation of the thermostable component.

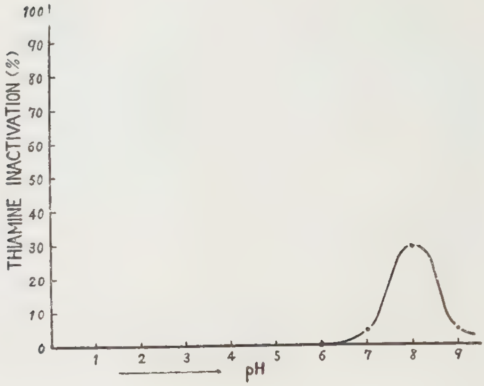

FIG. 4. Thiamine inactivation in nitrogen gas by boiled fern extract.

Expt. III. Dialyzability of Anti-thiamine Factors of the Fern Extract—To determine the dialyzability of the anti-thiamine factors, 20 ml. of fern extract introduced in a celophan sac were immersed overnight in 60 ml. of distilled water. Determination of anti-thiamine activity of the inner and outer solutions gave such results as shown in Table I.

The inner and outer solutions were boiled for 30 minutes at 100°, and examined for anti-thiamine activity at pH 6 and 8. The results are shown in Table II.

TABLE I

Thiamine Inactivation by the Dialyzed Fern Extract

	pH of medium	Thiamine inactivated
		γ
Inner solution*	6	3.5
	8	6.8
Outer solution*	6	0.0
	8	5.5

* Used amount of inner solution, 1 ml.; that of outer solution 3 ml.

The inner solution which inactivates thiamine at pH 6 and 8, presents no action at pH 6 after boiling for 30 minutes. As to the outer solution, however, which lacks the inactivating capacity at pH 6, the activity at pH 8 remains nearly unchanged even after heating.

Expt. IV. Effect of the Thiaminase Accelerants—The mechanism of thiaminase action was

TABLE II

Thiamine Inactivation by the Dialysed and Heated Fern Extract

	pH of medium	Thiamine inactivated
Boiled inner solution	6	0.0 γ
	8	7.0
Boiled outer solution	6	0.0
	8	4.5

TABLE III

Effect of Pyridine upon Anti-thiamine Activity of Fern Extract

	Pyridine*	pH of medium	Thiamine inactivated
Raw fern extract	+	6	116.9 γ
	−	6	13.2
	+	8	15.0
Boiley fern extract	+	8	15.3
	−	8	15.1
Inner solution	+	6	28.5
	−	6	3.4
Outer solution	+	8	6.0
	−	8	6.0

* Concentration of pyridine, 10^{-3} *M*. Amount of thiamine used in this experiments was 30 γ, except for 150 γ used in the experiment of raw fern extract.

clarified recently by Fujita *et al.* (*8*) who demonstrated that pyridine or anilin promotes the activity of thiaminase markedly, being enzymatically substituted for thiazole in thiamine.

Presence of such an accerelation was tested by adding pyridine to the fern extract, boiled extract, both inner and outer solutions resulting from dialysis of the extract. The results are shown in Table III.

The raw fern extract and the inner solution when added to thiamine solution with pyridine show a far increased anti-thiamine activity. When these solutions are mixed with sodium hydroxide and potassium ferricyanide and shaken with butanol, a green bluish fluorescence develops in the layer of butanol. Pyrichrome which is produced

from heteropyrithiamine*, a compound reported by Fujita (*8*), seems responsible for this fluorescence.

The addition of pyridine, however, to the boiled fern extract or outer solution presented no increase of antithiamine activity.

From the results of *Expt. I* to *Expt. IV*, it is evident that there are two different kinds of anti-thiamine factor in the ferns; namely, the thermostable and the thermolabile anti-thiamine factors. The properties of the respective factors may be summarized as below: 1) The thermostable anti-thiamine factor (an unknown substance): optimum pH at 8; active in the presence of oxygen; dialyzable; not promoted by addition of pyridine or anilin. 2) The thermolabile anti-thiamine factor (thiaminase): optimum pH at 6; active even in the absence of oxygen; non-dialyzable; promoted by addition of pyridine or anilin.

DISCUSSION

It was established by these experiments that the ferns possess two different kinds of anti-thiamine factors; namely, thiaminase and an unknown thermostable factor. Our ignorance as to this co-existence of two factors has been the barrier to the investigation of the anti-thiamine activity of the ferns. One of the reports (*4*) on the anti-thiamine activity of the ferns stated that thiamine inactivation proceeded in such a prompt velocity as comparable to an adsorption reaction. However, we have not encountered any instance of such a rapid inactivation in our serial experiments using the ferns growing in Japan. It was our finding that inactivating action of either thiaminase or thermostable factor occurs in a gradual progress. It has been known that these two factors are present not only in bracken fern, but in *Osmunda japonica* Thunb., *Equisetum arvense* L. and other kinds of fern.

Presence of factors resembling the thermostable anti-thiamine factor of bracken fern was found by us to prevail further among the plant kingdom; namely, spinach, beet greens, burdock, leaf of sweetpotatoes *etc.* (*9*) contain also a strong thermostable factor as that of bracken ferns. This supports the view that the thermostable anti-thiamine factors are almost universally distributed among the plants, and not limited to the ferns. The thorough biochemical investigation of these many thermostable anti-thiamine factors of the plants will take a long time. So far as we can say, the general features of these factors are that they are thermostable, resistant to drying, probably of rather small molecular weight, not an enzyme, active optimally at pH 8, and neces-

* 3-(2′-Methyl-4′-amino-pyrimidyl-5′)-methylpyridinium.

sitate oxygen for their action. However they may be differentiated by minor properties; for instance, the thermostable factor of bracken fern passes through celophan membrane whilst that of leaf of sweetpotatoes does not. As to the nature of the substance produced from thiamine by the thermostable anti-thiamine factors of the plants, we need a further investigation.

Hitherto "the Standard Table of Food Composition in Japan" dealt with bracken fern and royol fern as the vegetables containing no thiamine. On the other hand, shellfish possessing thiaminase has been found to contain thiamine. Expecting that there should be thiamine also in the bracken fern since it was found to possess thiaminase, we performed an experiment to detect thiamine in these ferns. The experiment demonstrated the presence in a considerable amount of thimaine in these plants as shown in Table IV. Such fact has been hitherto overlooked since thiamine, when extracted, is acted on by thiaminase intrinsic to them, and undergoes the decomposition process.

We have carried out the determination of thiamine roughly as follows: 5 g. of bracken fern leaves transferred to mortar containing 20 ml. of 10 per cent meta-phosphoric acid solution, are smashed sufficiently. This mixture is heated for 15 minutes at 80° and centrifuged at a high rate. The clear supernatant extract is neutralized to pH 4.5, diastase is added, and incubated overnight at 38°. Procedures hereafter follow the routine method by zeolite adsorption.

TABLE IV

Thiamine Content of the Ferns

	Thiamine content
	γ per g.
Pteris aquilina	0.5
Osmunda japonica Thunb.	0.7
Equisetum arvense L.	2.2

SUMMARY

The ferns possess two different kinds of anti-thiamine factors; namely, thiaminase and an unknown thermostable anti-thiamine factor. A biochemical study on these factors was carried out.

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THE METABOLISM OF CHOLIC ACID BY MICROORGANISM

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According to the observation made by Okada (*1*) cholic acid is subjected to a certain chemical change during the process of its circulation between intestine and liver. But the nature of the reaction product has not yet been worked out. The fact that cholic acid is oxido-reduced to some bile acids in the human body has been known, but the details of the change are not yet clarified either. According to the hypothesis propounded by T. Shimizu and co-workers, the cholic acid which is excreted into the intestinal canal is converted to other bile acid through ketone body by the action of intestinal bacteria, especially *B. coli*. The purpose of this paper is to present the results of experiments dealing with the change of dehydrocholic acid caused by the action of *B. coli*.

EXPERIMENTAL

Minced aseptic pancreases of five cows were poured into 2 *l.* of aseptic broth containing 21 g. of sodium dehydrocholate. At pH 8.0, *B. coli* was cultivated in the medium at 37° for 40 days in an incubator. The terminal pH of the medium was kept at 7.5 after cultivation. The cultivated solution was made sufficiently alkaline with sodium carbonate and then filtered. The filtrate was concentrated in a water-bath to 200 ml. and cooled. The NaCl produced was removed and the filtrate was extracted with ether under acidifying with HCl, and was separated into two parts: one ether soluble and the other insoluble. The ether-soluble part was washed with water and dried, the remaining ether being distilled off. In this way, a dark brownish jelly-likes substance was obtained. After removing lipid with petroleum-ether repeatedly, the material was dissolved in 200 ml. of 2 per cent ammonia solution and then 10 per cent barium chloride solution was added until no more precipitate was produced. The barium salt was separated into two parts: one soluble in water, and the other insoluble. To insoluble barium salt 10 per cent sodium carbonate solution was added, and the mixture was heated on a water-bath for 30 minutes, and then filtered. The filtrate was acidified with dilute HCl, upon which a dark, red, brownish precipitate was produced. The precipitate was filtered and dried. The dried precipitate was soluble in various

solvents, but no crystal was produced.

The filtrate from which water-insoluble barium salt was removed (barium salt water soluble fraction) was evaporated and was separated into 8 fractions according to the solubility. Each fraction was treated with 10 per cent sodium carbonate solution and the produced precipitate of barium carbonate was removed. The filtrate was acidified with HCl, and the precipitate produced was separated and dried. This precipitate was dissolved in ethyl acetate and left aside at room temperature. Powdery crystals were obtained from each fraction. The crystals of these fractions were collected and esterified with diazomethane. The methylester was dissolved in ethyl acetate and chromatographed over 100 g. of alumina. The crude crystal (m.p. 150-155°) was obtained. When recrystallized from ethyl acetate repeatedly, 607 mg. of crystal with m.p. 159° were obtained. The nitroprusside and Hammarsten reactions were negative. 250 mg. of the crystal were hydrolyzed with 2 per cent methanolic potassium hydroxide on a water-bath for 3 hours. The hydrolysate was acidified with HCl, the produced precipitate was filtered and dried. It was recrystallized from methanol-water repeatedly, and then column-like crystal with m.p. 218–219° was obtained. When the crystal was mixed with pure 3,7-dihydroxy-12-ketocholanic acid, the melting point was not lowered.

3,7-Dihydroxy-12-ketocholanic acid:

Analysis. Calcd. for $C_{24}H_{38}O_5$, C, 70.94; H, 9.36
Found C, 70.90; H, 9.21

Methyl 3,7-dihydroxy-12-ketocholanate:

Analysis. Calcd. for $C_{25}H_{40}O_5$, C, 71.43; H, 9.52
Found C, 71.27; H, 9.37

Ethanolic solution of the above methylester (300 mg.) was heated with 1 g. of hydroxylamine hydrochloride and 1.5 g. of anhydrous sodium acetate to make oxime. Recrystallized from acetone repeatedly, needle-like crystal (m.p. 108–109°) was obtained.

Analysis. Calcd. for $C_{25}H_{41}O_5N$, N, 3.22; Found N, 3.05

By the similar method, each filtrate of 8 fractions was collected and esterified with diazomethane. It was dissolved into ethyl acetate and chromatographed on alumina. By this procedual 300 mg. of methyl 3,7-dihydroxy-12-ketocholanate were obtained. From the filtrate, the solvent was distilled off and the residue was dissolved in 2 per cent methanolic potassium hydroxide and heated on a water bath for 3 hours. This hydrolysate was made acid with HCl, and the separated precipitate was extracted with ether. Following Wieland's method (*2*) the ether solution was extracted with 15 per cent HCl and 25 per cent HCl. A few crystal of 3,7-dihydroxy-12-ketocholanic acid was obtained from 15 per cent HCl fraction. But crystal was not obtained from 25 per cent HCl fraction.

SUMMARY

Mori (*3*) had confirmed in the culture medium of *B. subtilis* that only a ketone group of the 7-position of dehydrocholic acid was reduced

to an alcohol group. Shibuya (*4*), Yamasaki and Kyogoku (*5*) reported that if dehydrocholic acid was injected into a toad, 3(β)-hydroxy-7,12-diketocholanic acid was excreted in its urine. It seems probable, therefore, that only ketone group of the 3-position of the cholane nucleus could be converted to alcohol group in the toad body.

Takemoto (*6*) had proved that the ketone group of the 7-position of the bile acid nucleus was reduced to alcohol group and then to methylene group in putrefied media. The author found that ketone groups of the 3- and 7-positions of dehydrocholic acid were reduced into 3,7-dihydroxy-12-ketocholanic acid by *B. coli*. These observations indicate that among the ketone groups of 3-, 7-, and 12-positions of bile acid nucleus most vulnerable to the reducing action by *B. coli* is the one at 3-position and then that at 7-position, while the ketone group at a 12-position has strong resistance towards the bacterial action. Noteworthy is the fact that the ketone group at 3-position is reduced only to α-form by the action of bacteria.

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THE NITRATE UTILIZATION IN SEED EMBRYOS OF VIGNA SESQUIPEDALIS

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The process dominant in the nitrogen metabolism of bean seed embryos in the earlier stage of germination would be that of hydrolysis of reserve proteins in cotyledon. Resultant products of the hydrolysis then will be transported into the growing or anabolic regions of embryo. The reserve nutrients will soon be consumed, and inorganic nitrogen compounds, perhaps chiefly nitrate, absorbed from the soil might subsequently serve as the principal nitrogen source for the seedling plants.

Along the line of study in our laboratory on the physiology of sprouts of a legume, *Vigna sesquipedalis*, information on the change in activity of nitrate utilization not only spatial in both the anabolic and the catabolic organs but also temporal in the course of the germination stage* has urgently been needed.

The present paper will show a remarkable difference between the nitrate utilization in the catabolic organ, *i.e.* cotyledon, and that in the anabolic ones, *i.e.* plumule, hypocotyl, and radicle. Thus the latter might utilize nitrate as a starting material of protein synthesis, while in the former it might serve as a terminal hydrogen acceptor.

MAERIAL AND METHODS

Vigna sesquipedalis was used as the experimental material. With preliminary experiments a number of conventional bactericides were examined systematically as to their effectiveness of sterilization and injuriousness to growth of the legume seed. The following procedures were found to be most suitable.

Dry seeds were first treated for 90 minutes with a solution of hypochlorite of lime prepared as follows: 10 g. of commercial bleaching powder were well ground with 350 ml. of water and filtered; the filtrate (content of active chlorine: 5,000 p.p.m.) was employed. Then the seeds were thoroughly rinsed with sterile water to remove the remaining chlorine, and were sown on moist sea-sand in glass-pots of 14 cm. in diameter and 24 cm. in depth. About 1,500 ml. of sand were placed in each pot. The pots and

* As for the definition of the germination stage, compare that of Oota *et al.* (*1, 2*).

sand had previously been sterilized in a drying cabinet at 140° for 2 hours, and sterile water was poured into to wet the sand adequately. The seeds sown were covered with a thin layer (about 1 cm.) of sterile sea-sand, and then the pots were covered with a sterile glass-lid respectively. After designated times of incubation at 30° in the dark, the plants were harvested and selected for uniformity.

The plants so selected were cut with a sterile razor blade into four organs, *i.e.* plumules, hypocotyls, radicles, and cotyledons. Excised organs were washed with sterile distilled water to remove the sea-sand, and were submerged in a sterile KNO_3-sucrose solution (0.1 *M* KNO_3+0.1 *M* sucrose) in flasks with cotton stopper. After 5 hours incubation at 30° in the dark (" the absorption-period "), one half of the materials was removed for nitrate estimation. The remaining half was thoroughly rinsed with sterile distilled water to remove KNO_3 attached on the surface of the materials, and placed in a sucrose solution (0.1 *M*). It was followed by incubation for 20 hours at 30° in the dark (" the assimilation-period ") to allow the tissues to assimilate nitrate previously absorbed during the absorption-period. At the end of this period, remaining nitrate in the tissue was estimated. The amount of assimilated nitrate was obtained by subtraction of the amount of nitrate remaining in the tissue at the end of the assimilation period from that found at the beginning of the period. The " assimilated " nitrate obtained in this way naturally indicates only nitrate disappeared in the assimilation period, but not always the amount of nitrate really assimilated into organic nitrogen compounds such as amino acids *etc.*

Effect of the anaerobic conditions on the nitrate assimilation was studied as follows. The 4 day-old plants were employed. Conventional Thunberg tubes were used to obtain the anaerobic conditions. The excised organs which had previously been allowed to pass the absorption period were placed in a sterile sucrose solution (0.1 *M*) in the sterilized Thunberg tubes. Evacuation for 3 minutes by means of a vacuum-pump was followed by incubation in the dark at 30° for 20 hours. Effect of the anaerobic conditions on the nitrite production by cotyledon was studied in a similar way employing Thunberg tubes. The organs studied were obtained from 1 day-old seedling plants.

Total-, nitrate-, and nitrite-nitrogens were estimated as follows. The organs were well rinsed with sterile distilled water to remove the remaining medium, and mashed with 10 ml. of hot water in a mortar. Total nitrogen of macerates was measured by the method of Levy and Palmer modified by Yagi (*3*). For the nitrate nitrogen estimation, the macerate was boiled for 10 to 15 minutes to deproteinize, and was cleared by the addition of a small amount of charcoal powder. Nitrate nitrogen in a 5 ml.-aliquot of this clear solution was determined by the phenoldisulfonic acid method (*4*). Nitrite nitrogen was estimated with a modification of the Griess-reagent (*5*). In the nitrate as well as nitrite estimations a conventional electrophotometer was employed.

Unless otherwise stated, values on total nitrogen unit were calculated.

For the reasons of safety, sterility of the tissues employed was checked each time; a part of the tissues just before the commencement of the assimilation procedure was incubated in a sterilized KNO_3-sucrose solution (0.01 *M* KNO_3+0.01 *M* sucrose) for 20 hours at 30°. Nitrite was then analyzed colorimetrically. If nitrite was detectable

in the medium, it was supposed to be an evidence of bacterial contamination and the experimental results obtained with this group of materials were discarded.

Exceptionally, because of its own ability of nitrite accumulation (see below), sterility of cotyledons had to be certified bacteriologically. They were placed in a broth-agar medium and stirred well, and were incubated for 48 hours at 30°. If any bacterial colony appeared at the end of the incubation period, the results obtained with the lot from which the materials for the sterility-test were picked out were discarded.

For the examination of cytochrome spectra, intact organs as well as tissue homogenates were examined by an ocular spectroscope (Abbe's spectral ocular, Zeiss). As a reducing agent sodium hydrosulphite was employed.

RESULTS

Absorption and Assimilation of Nitrate in Excised Organs of Vigna Embryos—The ability of nitrate-absorption and -assimilation of excised organs, *i.e.* plumule, hypocotyl, radicle, and cotyledon, were investigated respectively. Representative results are shown in Tables I, II, III and IV. The term "assimilatory activity" in these tables denotes the ratio: assimilated nitrate per absorbed nitrate.

As illustrated in Fig. 1, drawn on the data collected from these

TABLE I

Absorption and Assimilation of Nitrate by Plumules Excised from Vigna Embryos of Various Ages

Age of tissue employed (*days*)	$\times 10^{-6}$ moles nitrate per mg. total N				Assimilatory activity[4] (*per cent*)
	Absorbed[1] (A)	Remained[2] (B)	Disappeared (A)–(B)	Found in medium[3]	
1	4.77	3.92	0.85	0.0	18
2	4.07	2.43	1.66	0.0	41
3	5.22	1.07	4.15	0.0	80
4	3.56	1.47	2.09	0.0	59
5	3.70	2.10	1.60	0.0	43
6	3.82	2.52	1.30	0.0	34

1) Found in the tissues after 5 hours incubation (absorption period) in a KNO_3 (0.1 *M*)+sucrose (0.1 *M*) solution in the dark at 30°.

2) Found in the tissues after 20 hours incubation (assimilation period) in a sucrose (0.1 *M*) solution in the dark at 30°.

3) Found in medium at the end of assimilation period.

4) See the text.

TABLE II

Absorption and Assimilation of Nitrate by Hypocotyls Excised from Vigna Embryos of Various Ages

Age of tissue employed (*days*)	$\times 10^{-6}$ moles nitrate per mg. total N				Assimilatory activity[4] (*per cent*)
	Absorbed[1] (A)	Remained[2] (B)	Disappeared (A)–(B)	Found in medium[3]	
1	6.64	5.10	1.54	0.0	23
2	9.82	7.08	2.74	0.0	28
3	7.91	5.01	2.90	0.0	37
4	5.84	4.22	1.62	0.0	28
5	6.80	5.10	1.70	0.0	25
6	7.01	5.75	1.26	0.0	18

1), 2), 3), 4). See the explanations of Table I.

TABLE III

Absorption and Assimilation of Nitrate by Radicles Excised from Vigna Embryos of Various Ages

Age of tissue employed (*days*)	$\times 10^{-6}$ moles nitrate per mg. total N				Assimilatory activity[4] (*per cent*)
	Absorbed[1] (A)	Remained[2] (B)	Disappeared (A)–(B)	Found in medium[3]	
1	4.06	4.05	0.01	0.0	0
2	6.60	6.30	0.30	0.0	5
3	7.55	4.65	2.90	0.0	38
4	13.30	3.55	9.75	0.0	73
5	8.76	3.52	5.24	0.0	60
6	8.50	3.83	4.67	0.0	55

1), 2), 3), 4). See the explanations of Table I.

tables, the ability of nitrate-absorption of hypocotyl and plumule each remains nearly constant throughout the germination stage. In respect of both radicle and cotyledon, however, the ability shows fairly remarkable change, that is to say, in radicle it has a sharp maximum on the 4th day of cultivation and in cotyledon it rises gradually with the age of the tissue. It should be remembered that in cotyledon, as an ever self-consuming reserve organ, an autolytic breakdown of the tissue con-

TABLE IV

Absorption and Assimilation of Nitrate by Cotyledons Excised from Vigna Embryos of Various Ages

Age of tissue employed (*days*)	$\times 10^{-6}$ moles nitrate per mg. total N			
	Absorbed[1] (A)	Remained[2] (B)	Disappeared (A)-(B)	Found in medium[3]
0	0.65	0.66	−0.01	0.00
1	2.08	2.07	0.01	0.01
2	2.80	2.78	0.02	0.02
3	4.91	4.61	0.30	0.29
4	7.06	5.44	1.62	1.60
5	14.90	5.10	9.80	9.71
6	16.50	6.35	10.15	10.02

1), 2), 3). See the explanations of Table I.

struction would proceed with age. This might result an increase in permeability of the tissue. As to radicle, the maximum absorption ability attained on the 4th day would assumingly be connected with abrupt appearance of lateral roots which means tremendous extention of absorption surface of the organ. From this point of view, it is of interest that the absorption ability of radicle falls rapidly after the 4th day. This will not be attributed to an unbalanced increase in the amount of total nitrogen in the later germination stage, since apparently the total nitrogen of radicle remains nearly constant in the later stage (*6*). It may be possible that the permeability of lateral roots decreases rapidly with maturation of the tissue.

The change in the assimilatory activity of excised organs is summarized in Fig. 2. It is noteworthy that the activity not always runs parallel with the absorption ability, but apparently with the respiratory activity of respective organs except cotyledon.*

In the experiments with the anabolic organs, *i.e.* plumule, hypocotyl, and radicle, practically no nitrate was detected in medium at the end of the assimilation period (see Tables I, II and III), hence the amount of nitrate "disappeared," *i.e.* the difference between the amount of nitrate "absorbed" and that "remained" can be supposed, with all probability, to be the amount of nitrate "assimilated." With re-

* Concerning the respiratory activity of respective organs, consult Oota (*2*).

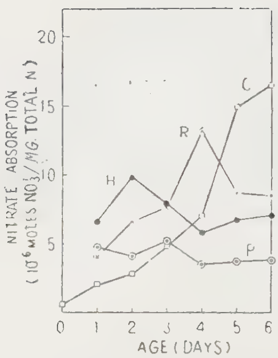

Fig. 1. Nitrate absorption by organs excised from *Vigna* embryos of various ages. P: plumule, H: hypocotyl, R: radicle, C: cotyledon.

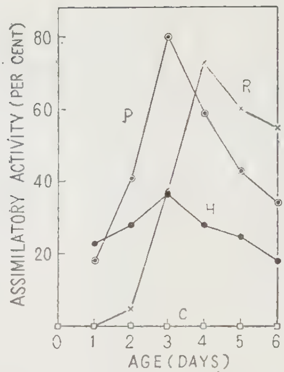

Fig. 2. Nitrate assimilation by organs excised from *Vigna* embryos of various age. P: plumule, H: hypocotyl, R: radicle, C: cotyledon.

gard to cotyledon, however, an unexpected discharge of "nitrate" from the tissue into medium was observed. Thus, as Table IV shows, practically whole amounts of nitrate "disappeared" from the tissue were always recovered in the medium. The "nitrate" liberation grows remarkable as the age of the tissue proceeds.

Since the estimates by the phenoldisulphonic acid method involve not only NO^-_3 but also NO^-_2, it should be suspected whether nitrate once absorbed in cotyledon was solely put out of the tissue, remaining unchanged. The nitrite production by cotyledon incubated in a nitrate-sucrose solution was examined colorimetrically. A representative result is shown in Table V and Fig. 3, where the values per embryo (or a pair of cotyledons) are described. Vigorous production of nitrite from nitrate by the cotyledon tissue is demonstrated in them. The younger the tissue, the more intense the production is. And the liberation of nitrite into the medium was found to take place mainly in the later germination stage. This should also be caused from the same physical conditions of the tissue as has been described above concerning the gradual increase in nitrate absorption by cotyledon.

Assimilation of Nitrate in Excised Organs of Vigna Embryos under Anaerobic Conditions—As has been shown in the preceding section, the assimilatory activity runs parallel with the respiratory activity, suggesting an interrelationship between the nitrate assimilation and the oxygen respiration.

TABLE V

Nitrite Formation by Cotyledons Excised from Vigna Embryos of Various Ages

Age of tissues employed (*days*) \ Medium	$\times 10^{-7}$ moles nitrate per a pair of cotyledons				
		KNO_3–sucrose[1] (A)	Sucrose[2] without KNO_3 (B)	Difference (A)–(B)	Total NO_2^- produced
0	In tissue	11.2	1.70	9.50	9.50
	In medium	0.0	0.00	0.00	
1	In tissue	12.3	1.70	10.60	10.60
	In medium	0.0	0.00	0.00	
2	In tissue	11.0	1.70	9.30	9.30
	In medium	0.0	0.00	0.00	
3	In tissue	2.77	0.78	1.99	3.99
	In medium	2.00	0.00	2.00	
4	In tissue	1.05	0.64	0.41	2.56
	In medium	2.15	0.00	2.15	
5	In tissue	0.90	0.50	0.40	2.45
	In medium	3.00	0.95	2.15	
6	In tissue	0.80	0.48	0.32	2.42
	In medium	2.50	0.40	2.10	

1) Incubated in a KNO_3 (0.1 *M*)+sucrose (0.1 *M*) solution for 5 hours in the dark at 30°.

2) Incubated in a sucrose (0.1 *M*) solution for 5 hours in the dark at 30°.

Hence the assimilatory activity under the anaerobic conditions (made by evacuation) was compared with that under the aerobic conditions. Table VI, which shows a representative result, indicates that in every anabolic organs studied the nitrate assimilation under the aerobic conditions clearly exceeds that under the anaerobic conditions.

Effect of evacuation on the nitrite formation in excised cotyledon was also examined (Table VII). In this case, the anaerobic conditions were found to somewhat favour the nitrite formation.

Observation of Cytochrome Spectra of Embryonic Tissues of Vigna Bean—It has been pointed out that the cotyledon tissue produces remarkable amounts of nitrite when it is in contact with nitrate. Thus the nitrate reduction in the tissue concerned appears to proceed no further than

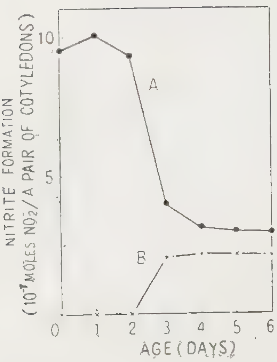

FIG. 3. Nitrite formation by cotyledons excised from *Vigna* embryos of various ages.

A: total NO_2^- produced, B: NO_2^- liberated in medium.

the step of nitrite. This suggests the exclusive role of nitrate reductase in the nitrate utilization of cotyledon. In the anabolic tissues, in which not a trace of nitrite could be detected when they were incubated with nitrate, absorbed nitrate should be converted or assimilated into further steps than nitrite.

On the other hand, Prof. T. Mori (*7*) in our laboratory, working with bacteria, has recently found a remarkable correlation between the cytochrome-type and nitrate reductase, that is to say, bacteria with nitrate reductase have cytochromes of the so-called *coli*-type (a_1, **b**) and cytochromes of the so-called *subtilis* type (a, b, c) would scarcely associated with the reductase. It will, therefore, be very interesting to examine cytochrome spectra of cotyledon in comparison with those of the anabolic tissues

TABLE VI

Effect of Anaerobic Conditions on Nitrate Assimilation in Organs Excised from Four Day-old Vigna Embryos

Conditions / Organs	Aerobic				Anaerobic*			
	$\times 10^{-6}$ moles nitrate per mg. total N			Assimilatory activity	$\times 10^{-6}$ moles nitrate per mg. total N			Assimilatory activity
	Absorbed[1] (A)	Remained[2] (B)	Disappeared (A)-(B)	*per cent*	Absorbed (A)	Remained (B)	Disappeared (A)-(B)	*per cent*
Radicle	10.55	4.87	5.68	51	10.55	7.02	3.53	35
Plumules	5.50	1.10	4.40	80	5.50	1.99	3.51	64
Hypocotyl	6.30	4.40	1.90	30	6.30	6.17	0.13	2

1) Found in the tissues after 5 hours absorption period in a KNO_3 (0.1 *M*) +sucrose (0.1 *M*) solution in the dark at 30°.

2) Found in the tissues after 20 hours assimilation period in a sucrose (0.1 *M*) solution in the dark at 30°.

* Evacuated Thunberg tubes were employed.

TABLE VII

Effect of Anaerobic Conditions on Nitrite Formation in Cotyledons Excised from One Day-old Vigna Embryos

Conditions / Medium	Aerobic			Anaerobic		
	$\times 10^{-7}$ moles nitrite per a pair of cotyledons					
	In tissue (A)	In medium (B)	Sum (A)+(B)	In tissue (A)	In medium (B)	Sum (A)+(B)
KNO_3-sucrose[1)]	12.0	0.0	12.0	16.03	1.40	17.43
Sucrose[2)]	1.7	0.0	1.7	3.46	0.80	4.26

1) Incubated in a KNO_3 (0.1 *M*)+sucrose (0.1 *M*) solution for 5 hours in the dark at 30°.

2) Incubated in a sucrose (0.1 *M*) solution for 5 hours in the dark at 30°.

* Evacuated Thunberg tubes were employed.

of *Vigna* embryos. Striking difference between these organs was found. Younger cotyledon (cut from embryo aged 2 days) which can produce nitrite vigorously contains cytochromes of *coli*-type, whereas every anabolic tissues show spectra of *subtilis*-type throughout the germination stage (Fig. 4). Most unexpectedly, cotyledon cut from older embryo (aged 6 days), which had lost almost whole ability of nitrite production, was found to have lost also the *coli*-type cytochromes; it showed cytochromes of *subtilis*-type instead.

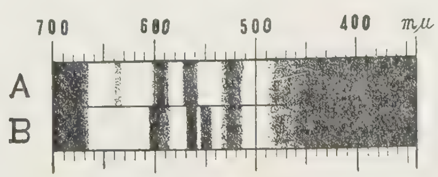

FIG. 4. Cytochrome-types.

A: *Coli*-type (a_1, **b**) ....Cotyledon in the earlier germination stage*.

B: *Subtilis*-type (a, b, c) ....Cotyledon in the later germination stage.

* It is as yet obscure whether the band a_2 (630–640 mμ), which is observed in *coli*-bacteria, is actually present in cotyledon tissue.

DISCUSSION

From the present investigations, it has become evident that the nitrate utilization by the seed embryo of *Vigna sesquipedalis* is quantitatively as well as qualitatively different from organ to organ. The most striking qualitiative difference was found between the anabolic organs and the catabolic one, that is to say, the former would be able to assimilate nitrate to the further steps than nitrite, while the latter can convert nitrate only into nitrite but not further.

That the nitrate assimilation in higher plants has an intimate relation to oxygen respiration has been suggested by Burström (*8*), Lundegårdh (*9*), and others. Lundegårdh has proposed a hypothesis that the energy required for the nitrate assimilation may be supplied from aerobic breakdown of carbohydrates. On the other hand, Shive (*10*) has shown that the nitrate assimilation in soybean is inhibited by higher concentration of oxygen. Recently, Nance (*11*) has reported an inhibitory effect of oxygen on the nitrate assimilation of wheat roots.

The results of the present investigations with seedling organs of *Vigna sesquipedalis* supports the conclusion of both Burström and Lundegårdh; oxygen respiration seems to play an essential role in the nitrate assimilation in the bean seedlings concerned.

As for the mode of nitrate utilization of cotyledon, nitrite as the product of probably one-step reduction of nitrate is subjected practically to no further transformation. It can therefore be concluded that in the cotyledon tissue nitrate reductase is the essential agent which can attack nitrate.

It is known that *Esch. coli* which is able to reduce nitrate to nitrite has cytochrome a_1 and **b**, and shows no activity of cytochrome oxidase, and *B. subtilis* which exhibits only little nitrate-reducing power possesses cytochrome a, b and c, and, as is consequently expected, shows intense activity of cytochrome oxidase. Moreover, high activity of nitrate-reductase seems to associate with the *coli*-type of cytochrome, while negligibly weak capacity of nitrate reduction and the *subtilis*-type of cytochrome are always accompanied with each other (*7*).

Recently it has been found in our laboratory (*7*) that the cytochrome type of normal *B. pumilus* (essentially *coli*-type) and that of its mutant (*subtilis*-type) were interconvertible under appropriate conditions. Moreover, the change in the cytochrome-type was also found to be necessarily accompanied with that in the activities not only of cytochrome

oxidase but also of nitrate reductase.

The present investigations have revealed that cotyledon at the earlier stage of germination when highly active nitrate reductase would function, possesses cytochromes of *coli*-type, while later as the reductase action decays cytochromes of *subtilis*-type appear instead in the tissue in question. This means a remarkable fact that also in the cotyledon tissue of *Vigna sesquipedalis* the *coli*-type cytochrome could be transformed into the *subtilis*-type. On the other hand, it was also found in our laboratory (*12*), that in the earlier germination stage cotyledon shows rather weak activity of cytochrome oxidase which grows up gradually with the age of the organ.

These observations together indicate circumstances of exactly similar nature to that described about bacteria, and would imply a probable role of nitrate reductase in the physiological function of cotyledon. Thus it appears likely that nitrate reductase may serve as a terminal oxidase in the respiratory mechanism of cotyledon at least in the earlier stage of germination, just as shown with regard to the respiration mechanism of *Esch. coli* (*13, 14*).

SUMMARY

The nitrate absorption and the nitrate assimilation by excised organs of seed embryos of *Vigna sesquipedalis* grown in the dark were investigated. The most vigorous absorption was found in radicle, especially aged 4 days when lateral roots appeared. As for nitrate assimilation in the dark, plumule and radicle showed strong activity, and hypocotyl relatively weak one. The nitrate assimilation in these organs kept pace with the oxygen respiration, and under anaerobic conditions the assimilation was greatly diminished.

In cotyledon absorbed nitrate was converted into nitrite, but not further. The nitrite production decreased as the age of tissue progressed, when a greater part of the nitrite accumulated in the tissue was put out into medium. In the early stage of germination the cotyledon tissue was found to have cytochromes of *coli*-type (a_1, **b**), and in the later stage that of *subtilis*-type (a, b, c). A possiblity was pointed out that nitrate may serve as a terminal hydrogen-acceptor in the cotyledon tissue at least in the earlier stage of germination.

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STUDIES ON SOME NEW METABOLIC PRODUCTS OF PENICILLIUM. II*

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The author isolated two yellow crystalline metabolic products from the metabolite solution of *P. amarum nov. sp. Munekata* which was obtained in a sugar manufacturing factory in Formosa.** The author gave the name "*pinselin*" and "*pinselic acid*" to those pigments and reported on the general properties and some derivatives of the former in the previous paper (*1*). The present paper deals with the chemical structure of pinselin as well as pinselic acid.

Pinselin crystallized in golden yellow long prisms, m.p. 225°, and it gave a stable deep green color with $FeCl_3$. Its molecular formula, $C_{16}H_{12}O_6$, was proposed from the results of elementary analysis and molecular weight determination. The Zeisel's method showed the presence of one methoxyl group and the reaction for methylene oxide with phloroglucinol and H_2SO_4 was found to be negative. By alkylation, benzoylation, and acetylation, all the hydroxyl groups responsible for the color reaction with $FeCl_3$ were substituted, giving dimethyl-, dibenzoyl-, and diacetyl-pinselins, respectively; but it is worthy to note that no hydroxyl group was readily methylated by dimethylsulfate. It was clarified that pinselin contained neither free carboxyl group nor active carbonyl group, since it was neutral and extractible with ether in aqueous Na_2CO_3 solution and did not react with any of phenylhydrazine, 2,4-dinitrophenylhydrazine, semicarbazide and hydroxylamine. Pinselin remained unchanged when heated with 30 per cent aqueous NaOH solution at 100° for 5 hours, while it gave pinselic acid on boiling with a concentrated sodium

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* This work was carried out in the years of the World War II and was reported in the meetings of the Agricultural Chemical Society of Japan in July, 1943 and in March, 1944 and the publication of this paper has been detained by the restricted circumstances of the said Society after the war.

** Kindly shared by Dr. K. Sakaguchi. Its morphological characteristics were previously reported by the author in the meeting of the Agricultural Chemical Society of Japan (April, 1944).

ethylate solution. It was presumed that pinselin had at least one terminal methyl group inasmuch as it gave one molecule of acetic acid as an ultimate oxidation product by chromic acid in concentrated phosphoric acid solution. On decomposition with concentrated iodic acid, pinselin yielded a product, $C_{14}H_{10}O_4$, which was an easily sublimable neutral compound, and the same substance was also obtained from pinselic acid when it was heated at 200°. Pinselic acid had apparently double melting points of 200° and 250–252°, but it was ascertained that pinselic acid was decomposed at the first melting point under liberation of one molecule of CO_2, giving rise to the above-mentioned compound, $C_{14}H_{10}O_4$. Pinselic acid contained no methoxyl group and the result of elementary analysis supported $C_{15}H_{10}O_6$ for its empirical formula. It gave a stable green color with $FeCl_3$ similarly to pinselin and on methylation by diazomethane, it gave a trimethylate, which showed no melting point depression when mixed with dimethylpinselin. On acetylation it gave a diacetylderivative of m.p. 207° but the mixed melting point of this acetylate with diacetylpinselin (m.p. 207°) showed a remarkable depression. Pinselic acid is an acidic compound and not extracted from aqueous $NaHCO_3$ solution with ether.

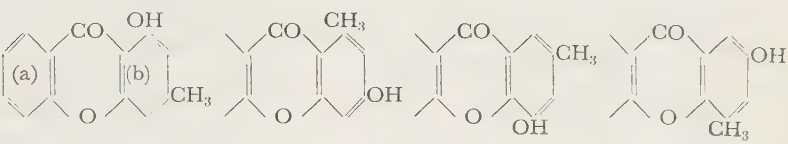
On the basis of these facts the following résumé was obtained:— Pinselin has one hardly saponificable carbomethoxyl group and pinselic acid is the corresponding free carboxylic acid. This carboxyl group has a characteristic to be readily removed merely by heating at 200°, while the elimination of the carbomethoxyl group of pinselin needs cooperation of concentrated mineral acid, and as the result of decomposition, the same product, $C_{14}H_{10}O_4$, is obtained in both cases. Of six oxygen atoms of pinselin, two are found being as carbomethoxyl, two as phenolic hydroxyls and the rest as inactive. Pinselin has at least one terminal methyl group in its molecule.

The carboxyl group of pinselic acid can be readily methylated by diazomethane, while it is hardly esterified by HCl-methanol, showing a yield of only about 7 per cent after 5 hours' boiling with anhydrous HCl-methanol.

The above-mentioned decomposition product, $C_{14}H_{10}O_4$, crystallized in yellow needles, m.p. 253°, giving a green color with $FeCl_3$, and yielded a diacetyl derivative on acetylation, showing that the original two hydroxyl groups of pinselin were retained, but it called some attention that one of those had become very resistant to methylation by diazomethane. On potash fusion, the author isolated orcinol from the neutral

fraction, and only a very small amount of the white acidic compound (m. p. 189°) besides much of oxalic acid from the acidic fraction. Unfortunately the quantity of this compound was too small to be examined further, but it was presumed to be gentisic acid on the basis that its aqueous solution gave a deep blue color with $FeCl_3$ and reduced ammoniacal $AgNO_3$ solution, and that the melting point of the admixture with synthesized authentic specimen (m.p. 200°) was 197°.

Considering that two oxygen atoms in pinselin are inactive and pinselin gives a similar ultraviolet absorption spectrum to that of xanthone, and a phenol derivative and phenol-carboxylic acid are produced on potash fusion of $C_{14}H_{10}O_4$, the author presumed a xanthone ring as the nucleus of pinselin, and so far as that is, the combining ways of orcinol must be any of;



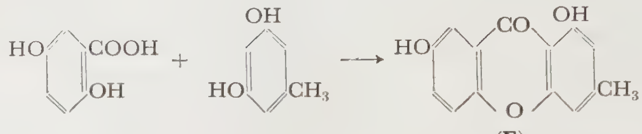
(A) **(B)** **(C)** **(D)**

The fact that pinselin is very stable for alkali suggests the presence of one of hydroxyls on the (a)-ring and its most probable position in accordance with production of gentisic acid on potash fusion is the 7-position. On the other hand, the fact that one of the hydroxyls of the decarboxylated product from pinselic acid resists against the methylation leads to take the assumption that this hydroxyl is attached probably to the 1- or 8-position, and thus the author considered that the most probable constitution of $C_{14}H_{10}O_4$ is (**A**), having a hydroxyl in the 7-position, which was ascertained by its tentative synthesis as below.

Orcinol and synthesized gentisic acid were directly condensed in the presence of anhydrous zinc chloride by the method of Kostanecki and Nessler (*2*); and the product was purified and the yellow needle crystals obtained were examined (m. p. 253°). The result of analysis supports $C_{14}H_{10}O_4$; the color reaction with $FeCl_3$ and characteristics of diacetyl and monomethyl derivatives (dimethyl derivative was not obtainable also in this case) were quite similar to those of the decomposition product of pinselin by iodic acid; and the melting point of admixture showed no depression. Kostanecki studying the synthesis of xanthones from resorcinol and various derivatives of salicylic acid, ascertained that all of the hydroxyxanthones obtained had hydroxyls in the 8-posi tion, but not in the 6-position, proving the stronger reactivity of the

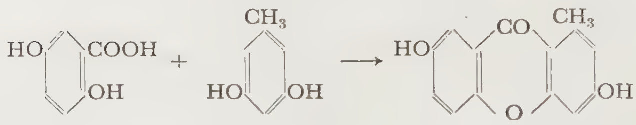
hydrogen atom attached to the carbon atom between two hydroxyl groups of resorcinol; and from this fact it is analogously concluded that the reaction of orcinol and gentisic acid must take place as the schema of (I), by which the resistency of one of two hydroxyls of the product to the methylation is explained without consistency (*6*), and thus the structure of the decomposition product, $C_{14}H_{10}O_4$, has been decided as (**E**).

I



(**E**)

II.

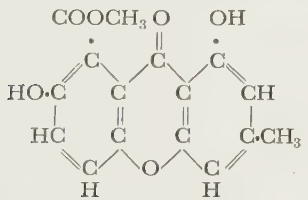


(**F**)

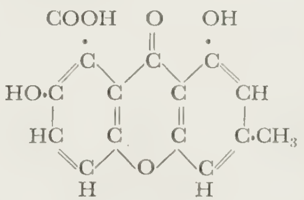
All attempts to obtain carbomethoxyl-containing decomposition products from pinselin and its derivatives by chromic acid, $KMnO_4$, HNO_3, H_2O_2 or MnO_2 under various conditions have been hitherto unsuccessful, and in all cases they were recovered unchanged, unless deeply decomposed. On mild potash fusion, none but pinselic acid or $C_{14}H_{10}O_4$ was obtained, and the treatment by alcoholic potash did not result in any derivative of benzophenone. However, the carboxyl group of pinselic acid, as mentioned above, can be readily removed by mere heating at 200° and very hardly esterified by alcoholic HCl. Such properties of a carboxyl radical are characterized by the accumulation of negative groups or the presence of substituted radicals in both *ortho*-positions to a carboxyl radical. On the other hand, it is well known that the hydroxyl of the *ortho*-position to a carbonyl group in hydroxy-xanthones can be hardly methylated. If the carbomethoxyl group is presumed to be located in the 2-position of decarbomethoxylated pinselin, there comes a contradiction to the fact that all the hydroxyls of pinselin and pinselic acid can be easily methylated by diazomethane, since the hydroxyl in the 1-position would be more resistant to the methylation. On the contrary, the presence of carbomethoxyl in the 8-position is supported by all these relations, and especially by the fact that the hydroxyl of the 1-position of decarbomethoxylated pinselin strongly resists to methylation by diazomethane otherwise than in pinselin

or pinselic acid. Herzig (*3*), studying the methylation of quercetin by diazomethane, confirmed that the effect of steric hindrance of the hydroxyl group in the *ortho*-position on the carbonyl group of a pyrone ring presented itself after the other hydroxyls had been completely methylated beforehand. Furthermore, it was pointed out by Herzig and Stanger (*4*) that the effect of steric hindrance of carbonyl group upon the hydroxyl in the 1-position of a xanthone ring can be excluded by the presence of co-existing hardly methylatable hydroxyl groups. These facts correspond with that which in pinselin and pinselic acid the hindering effect of the carbonyl group on the hydroxyl in the 1-position is removed by the inactivation of the hydroxyl in the 7-position under the influence of the carboxyl in the 8-position and mannifests itself after decarboxylation.

Summarizing the facts above-mentioned, the author advanced the following structures for pinselin and pinselic acid:



Pinselin



Pinselic acid

Although pure crystals of pinselin and pinselic acid are both very hardly soluble in water, their yields were always much greater from the metabolite solution than from the mycelium, and the yields of pinselin and pinselic acid were, respectively, about 10 mg. and 2 mg. per liter of the solution. Of the carbon sources tested, sucrose was the best, and dextrose, levulose or the mixture of them was also considerably suitable but always giving much less yield of the pigments. Nitrogen source used was lacto-peptone which gave the best yield of these pigments in combination with sucrose; and inorganic nitrogen source, NH_4-tartarate or asparagine was almost worthless for the production of the pigments. Considerable growth of this mold was shown on the peptone solution without any addition of carbon source, but there was observed no production of these pigments. It was, of course, suspected that pinselic acid might be derived from pinselin in the course of isolation from the medium but this suspicion was excluded from the fact that the yield of the pinselic acid did not show any increase when pinselin

was added to the filtrate of metabolite medium and recovered.

Pinselin can be regarded as a derivative of a condensation product of gentisic acid and orcinol or intramolecular dehydration product of 2,2′-dihydroxybenzophenone derivative. In this view it is of some interest to note that Raistrick and Simonart (*5*) isolated gentisic acid from the metabolite solution of *P. griseo-fulvum*; and Nishikawa (*7*) obtained "Sulochrin" from the mycelium of *Oospora-sulfureaochracea*, and moreover the methyl radicals of sulochrin, pinselin and "Ravenelin" (*8*) which is the only xanthone derivative structurally elucidated as a mold metabolic product, are all in the *para*-position to the carbonyl group.

EXPERIMENTAL

Isolation of the Pigments—After *P. amarum* was cultivated at 30° for 30 days on the medium containing sucrose (100 g.), lacto-peptone (7.5 g.), K_2HPO_4(1 g.) and $MgSO_4 \cdot 7H_2O$ (0.33 g.) in 1 *l.* of distillated water, the metabolite solution was filtered and its pH was adjusted to 3.0 with dilute HCl. The precipitate formed was dried and extracted with ether, and the extract was shaken with 5 per cent aqueous $NaHCO_3$ solution. From the ether layer pinselin was isolated as mentioned in the previous paper(*1*) and the aqueous layer was treated as below. After acidifying the solution, the precipitate formed was dried and extracted with hot benzene repeatedly. Then benzene solution was concentrated to about one-third of the volume and the brown resinous substance formed was removed by decantation. After adding about the same volume of ligroin, the precipitate formed was removed again by filtration. About twice volume of ether was added to the filtrate and it was kept in a desiccator in an ice-box. Then the crude crystals of pinselic acid obtained were recrystallized from ethylacetate and ligroin.

General Properties and Analyses of the Pigments—Pinselic acid crystallizes in long yellow needles and gives green color with $FeCl_3$ in ethanol. It decomposes at 195–200° and after solidifying, remelts again at 250–252°. Almost insoluble in water, petroleum ether and ligroin, moderately soluble in methanol, ethanol, chloroform and ethylacetate and readily soluble in acetone and pyridine. The Zeisel's method shows absence of methoxyl group.

Analysis: Calcd. for $C_{15}H_{10}O_6$ C 62.90%; H 3.50%
Found, C 63.15%; H 3.56%

Some Derivatives of the Pigments—

Diacetylpinselic Acid: It was prepared by the usual method with acetic anhydride and Na-acetate, and recrystallized from absolute alcohol. Colorless, transparent plates, m.p. 207°.

Analysis: Calcd. for $C_{15}H_8O_6$ $(CH_3CO)_2$, C 61.41%; H 3.78%
Found, C 61.41%; H 3.83%

Soluble in ethylacetate, hot methanol, ether and pyridine, hardly soluble in cold

methanol and benzene, and insoluble in petroleum ether and ligroin. When it is warmed with 5 per cent aqueous NaOH solution for a few minutes it is readily saponified to pinselic acid.

Trimethylpinselic Acid: 83 mg. pinselic acid dissolved in 6 ml. of 10 per cent aqueous NaOH solution was treated twice with each 0.3 ml. of dimethylsulfate, then 5 ml. of 10 per cent NaOH solution were added and the mixture was heated at 100° for 15 minutes. After cooling to 80°, 0.5 ml. of dimethylsulfate was added and the whole was again heated in the same manner. After cooling, it was acidified with HCl and filtered. The yellow precipitate thereby obtained, which was difficult to crystallize, was suspended in ether and shaken with an ether solution of diazomethane. After driving the solvent off, the residue was recrystallized from ethylacetate and ligroin. Yield, 80 per cent. Yellow cubic crystals, m.p. 212.5° and the melting point of the admixture with dimethylpinselin showed no depression. When pinselic acid was almost dissolved in anhydrous ether and treated with a large excess of diazomethane twice keeping each for a week at room temperature, the same product was obtained but the yield was 57 per cent.

Analysis: Calcd. for $C_{15}H_7O_3(OCH_3)_3$, C 65.85%; H 4.88%
Found. C 65.35%; H 4.69%

Esterification of Pinselic Acid—100 mg. of pinselic acid dissolved in 10 ml. of anhydrous methanol was refluxed for 5 hours with dried HCl gas passing through it. After expelling the solvent, the mixture was extracted with ether. The extract was washed with dil. aqueous $NaHCO_3$ solution, dehydrated with Na_2SO_4, and then evaporated. The residual yellow powder was put to recrystallization from ethanol. Yield 7 mg. Long yellow prisms, m.p. 225,° and the melting point of the admixture with pinselin showed no depression. From the acidic fraction 80 mg. of pinselic acid was recovered.

Oxidation of Pinselin by Chromic Acid—213 mg. of pinselin was refluxedwith anhydrous chromic acid (12 g.), $K_2Cr_2O_7$ (3 g.), 90 per cent H_3PO_4 (20 ml.), and 30 ml. of water on a boiling water-bath for 4.5 hours, and then the liquid was submitted to steam distillation. An aliquot of the distillate was titrated with alkali, and the remaining was used to prepare a *p*-bromophenacylester by the usual method. The melting point of the obtained ester was 85°, and that of the mixture with the authentic specimen of *p*-bromophenacylacetic acid showed no depression. The distillate consumed 14.82 ml. of $N/20$ NaOH. (Calcd. for 1 mole acetic acid: 14.20 ml.).

Decomposition of Pinselin by Iodic Acid—108 mg. of pinselin was heated with 2 ml. of concentrated iodic acid at 130–140° for 15 minutes, subsequently at 155° for 45 minutes. The yellow crystalline precipitate formed was taken on a glass filter and washed successively with water, Na thiosulfate and water until the alcoholic solution of the precipitate did not give the iodine-reaction by Ag ions and recrystallized from ethanol. Yellow needles; m.p. 253°. The same product was obtained by heating of 100 mg. of pinselin with 1 ml. of H_2SO_2 at 200° for 15 minutes.

Analysis: Calcd. for $C_{14}H_{10}O_4$, C 69.42%; H 4.13%
Found, C 68.82%; H 4.04%

Decomposition of Pinselin by Sodium Ethylate—103 mg. of pinselin was refluxed with 15 per cent sodium ethylate for 3 hours. After cooling 20 ml. of water, was added

to the reaction mixture and concentrated to one-third volume *in vacuo*. The brownish yellow precipitate, which was given by making the concentrate acidic with HCl, was suspended in 10 per cent aqueous $NaHCO_3$ solution and extracted with ether. On evaporating the extract after dehydration and on recrystallizing the residual yellow solid from ethanol, 15 mg. of pinselin was recovered. The residual aqueous solution after extracting with ether was filtered from brown precipitate and exhaustively washed with 10 per cent aqueous $NaHCO_3$ solution. The combined liquid of the filtrate and washings was acidified with HCl and shaken with ether. The ether extract was evaporated after dehydration and the residual solid was recrystallized from ethylacetate and ligroin. Yellow needle crystals having all the properties of pinselic acid were obtained. The decomposition point (200°) of the admixture of authentic pinselic acid and a mixed melting point of its acetylderivative (m.p. 207°) with diacetylpinselic acid showed no depression.

Decomposition of Pinselic Acid by Heating—After the air in a V-shaped combustion tube containing finely powdered pure pinselic acid at the bottom was replaced with nitrogen gas washed with H_2SO_4 and KOH, the lower part of the tube containing the sample was gradually heated in a glycerol bath, passing the nitrogen gas into the tube during the procedure, and the mixture of gases developed was passed through a potash bulb. An abrupt decomposition followed by a partial sublimation took place at 205° of bath temperature. After completion of the reaction at this temperature, the contents were cooled in the stream of nitrogen gas. 15.0 mg. of CO_2 was thus developed from 102.0 mg. of pinselic acid. (Calcd. for 1 mole of CO_2: 15.7 mg.) The sublimate and the remaining solid were recrystallized from hot ethanol. Yellow needles. (m.p. 253°.) A mixed melting point with the above-mentioned decomposition product, $C_{14}H_{10}O_4$, from pinselin showed no depression.

Acetylation of the Decomposition Product, $C_{14}H_{10}O_4$, from Pinselin—After a mixture of 50 mg. of pinselin and 1.1 ml. of acetic anhydride was boiled in the presence of anhydrous sodium acetate, the mixture was poured into ice-water, the white precipitate then formed was filtered and recrystallized from absolute alcohol. Mica-like lustrous white foliar crystals, m.p. 171°.

Analysis: Calcd. for $C_{14}H_8O_4(CH_3CO)_2$, C 66.25%; H 4.33%
Found, C 66.30%; H 4.27%

Methylation of the Decomposition Product, $C_{14}H_{10}O_4$, from Pinselin— The sample was dissolved in anhydrous ether and treated twice with a large excess of diazomethane. The product was recrystallized from ethanol. Yield, 92 per cent. Pale yellow needles, m.p. 148.5°.

Analysis: Calcd. for $C_{14}H_9O_3(OCH_3)$, C 70.31; H 4.69%
Found, C 70.35; H 4.54%

Even when the sample was treated with dimethylsulfate in the same way as mentioned in the preparation of trimethylpinselin, dimethyl detrivative was not obtained.

Potash Fusion of the Decomposition Product, $C_{14}H_{10}O_4$, from Pinselin—To the mixture of 200 mg. of $C_{14}H_{10}O_4$, 800 mg. of solid KOH and 0.05 ml. of water in a pyrex flask, 0.5 ml. of acetone was added to bring to solution. After expelling acetone at 40–60°, the flask

was gradually heated in an oil-bath, passing nitrogen gas through it, and the bath-temperature was brought up to 270° in one hour. After retaining at this temperature for 15 minutes, the flask was quickly cooled and then 10 ml. of water and 10 ml. of concentrated sulfuric acid were added, successively. The mixture was filtered and the filtrate was extracted with ether. The extract was kept in an ice-box, the brown resinous substance deposited was removed by decantation and the clear solution was shaken with 5 per cent aqueous $NaHCO_3$ solution. The aqueous fraction was shaken with ether after acidification and the ether extract was washed with aqueous $CaCl_2$ solution, then dehydrated with Na_2SO_4 and evaporated. The residual yellowish powder was put to recrystallization from warm water by using some charcoal, but its quantity was too small to be recrystallized repeatedly that the almost white homogeneous powder thereby obtained was examined. It gave a deep blue color with $FeCl_3$ and reduced ammoniacal $AgNO_3$. Its melting point was 189° and the melting point when it was mixed with the synthesized gentisic acid (m.p. 200°) was 197°.

The above-mentioned ether fraction was evaporated and the residue was recrystallized from a small quantity of water, using some charcoal. The colorless prisms obtained showed all the properties of orcinol (m.p. 107°, sweetness, dark purple color with $FeCl_3$ and deep red coloration when its chloroform solution is warmed with KOH) and gave no depression of melting point when it was mixed with the authentic specimen of orcinol.

Analysis: Calcd. for $C_7H_8O_2$, C 67.74%; H 6.50%
Found, C 67.31%; H 6.53%

Synthesis of 3-Methyl-1,7-dihydroxy-xanthone—Gentisic acid was prepared from hydroquinone, glycerol (sp. gr. 1.26) and $KHCO_3$ by heating them together at 170–185° (bath temperature) for 12 hours in the atmosphere of CO_2. A mixture of 770 mg. of gentisic acid, thus obtained, and anhydrous $ZnCl_2$ was heated at 115–120° (bath temperature) for 4 hours under a $CaCl_2$-tube. After cooling the products were washed with cold water and the remaining yellow solid (165 mg.) was recrystallized several times from absolute ethanol. Yellow needles, m.p. 253°, yield 25 mg.

Analysis: Calcd. for $C_{14}H_{10}O_4$, C 69.42%; H 4.13%
Found, C 68.99%; H 4.00%

A mixed melting point with the decomposition product, $C_{14}H_{10}O_4$, from pinselin showed no depression. Its acetate and methylate were prepared, and their mixed melting points with the corresponding derivatives of $C_{14}H_{10}O_4$ also showed no depression.

The author is deeply indebted to Dr. Sakaguchi, Dr. Sumiki and Dr. Asai for their constructive suggestions to perform this work. Further thanks are due to the staffs of the Agricultural Department and the Pharmaceutical Department of the Tokyo University and of the Institute of Scientific Research for their kind helps to take absorption spectra and to carry out elementary analyses. Appreciation is also expressed to the Dai Nippon Beer Co. Ltd. for the aid to carry out this work.

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THIOL CONTENT AND SUCCINOXIDASE ACTIVITY OF ISOLATED RAT LIVER MITOCHONDRIA

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It is now usually assumed that -SH groups play a vital role in certain enzymatic activities. In this line extensive works were carried out by Barron and his coworkers (*1*, *2*) using oxidizing, mercaptide-forming and alkylating reagents, all of which are capable of blocking or destroying -SH group. One of the authors (*3*) also reported that by bloking -SH group of succinodehydrogenase its activity of coupled oxidative phosphorylation was stopped. More recently, Kun (*4*) reported that succinodehydrogenase was chiefly located in mitochondria of tissue cell and its activity was lost when -SH group of mitochondria was blocked by methylglyoxal. To get more intimate knowledge of the participation of -SH group of mitochondria in succinodehydrogenase system, the following studies were carried out, in which the experiments were extended partly to α-ketoglutaric oxidase.

EXPERIMENTALS

Mitochondria were isolated by the procedure of Kennedy and Lehninger (*5*). Rat liver was ground in 0.88 *M* sucrose solution. After removal of intact cells, nuclei and red cells *etc.*, the mitochondria were agglutinated by the addition of KCl, and precipitated by centrifugation, followed by three succesive washings with 0.15 *M* KCl. The mitochondria were finally resuspended in 0.15 *M* KCl so as to make 1 ml. of the suspension correspond to 0.75 g. of original fresh liver.

Succinoxidase was assayed manometrically according to Schneider and Potter (*6*), and α-ketoglutaric oxidase by using Lehninger's mixture (*7*). The components of the reaction mixture for the former was described in Fig. 1. and that for the latter in Fig. 3. The addition of cytochrome c seems to be essential in so far as it promotes the oxygen uptake of mitochondria to a considerable extent.

As soon as the manometric determination was finished the content of vessel was transferred into a conical tube and centrifuged. The supernantant was poured off and the residue was resuspended in 3 ml. of cold distilled water. On this solution determination of -SH was carried out by two methods, namely, ferricyanide method (*8*, *9*)

using 2 ml. of the suspension, and amperometric titration method (*10–13*) using 1 ml. Both gave a quite concordant value. For the amperometric method, the titrated amount of solution was reduced to 8 ml. and moreover it contained 50 per cent ethyl alcohol, so it was possible to determine as small a quantity as 0.1 μM–0.5 μM of -SH groups. Ethyl alcohol seems to make -SH groups of mitochondria reactive, since without it no consumption of $AgNO_3$ was detectable.

When the reactivation with reduced glutathione was attempted, GSH was added 10 minutes after mitochondria was made contact with thiol reagent, and the determination of -SH group was carried out after washing mitochondria with cold 0.15 *M* KCl solution.

RESULTS

Succinoxidase Activity and -SH Content

Mercaptide-forming Agents—Inhibition studies were made using some mercaptide-forming agents including heavy metals such as *p*-chloromercuribenzoate (PCMB), $HgCl_2$, $CuSO_4$, $CdSO_4$ and $ZnSO_4$. The results are illustrated in Fig. 1.

As shown in Fig. 1., the curves of percentage decrease of oxygen consumption with increasing concentration of the inhibitors run quite parallel to the similar curve of -SH contents in the case of PCMB, Hg^{++} and Cu^{++}, while this is not the cases with Zn^{++} and Cd^{++}, which show low affinity for mitochondrial -SH group.

Oxidizing Agents—The effect of oxidizing agents such as tetrathionate, forricyanide and chloropicrin are illustrated in Fig. 2. The last mentioned substance might be regarded as the oxidizing agents, since the inhibition by it can be relieved by means of GSH as described later. Here also quite excellent consistency was demonstrated between enzymatic activity and -SH content in the cases of tetrathionate and chloropicrin. But this is not the case with ferricyanide due to its sluggish reactivity toward mitochondrial -SH as revealed by the experiment.

Alkylating Agents—The effect of alkylating agents such as iodoacetamide, methyl-bis (β-chloroethyl) amine and chlorovinyl-methylketone* are shown in Table I.

Iodoacetamide exhibited unexpectedly both low inhibition of O_2-uptake and low reactivity to -SH groups of mitochondria, such that in the final concentration as high as *M*/60, only 21 per cent inhibition of

* As stated in previous report (*3*) this new -SH reagnets, like chloropicrin, inhibited succinoxidase far more strongly than endogenous respiration, the reverse being observed in iodoacetic acid.

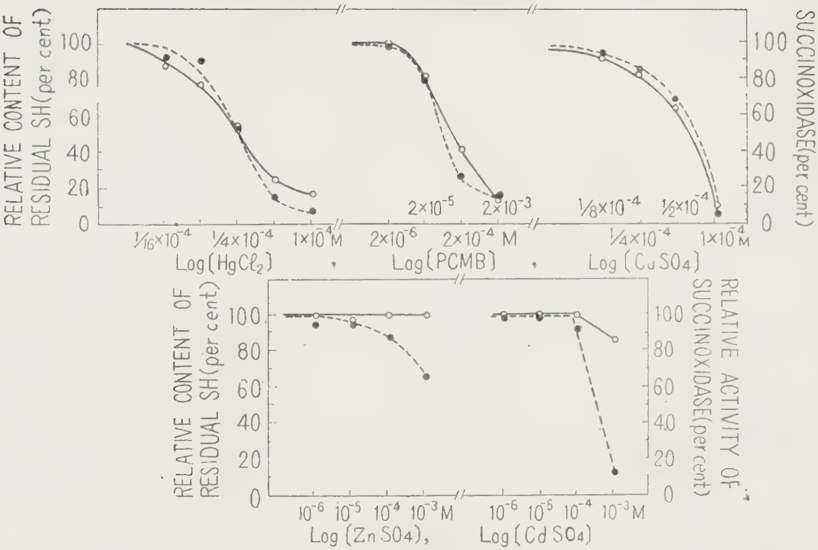

FIG. 1. Effect of mercaptide-forming agents on succinoxidase and -SH content of mitochondria.

Residual -SH content ——o——o——

Succinoxidase activity - - -•- - -•- - -

The control values without inhibitors are represented as 100 per cent. The reaction mixture of enzyme assay: water or solutions of inhibitors in different concentrations, 0.5 ml.; 0.1 *M* phosphate buffer (pH 7.4), 1.0 ml.; 0.5 *M* succinate, 0.30 ml.; 1×10^{-4} *M* cytochrome c, 0.40 ml.; 4×10^{-3} *M* $CaCl_2$, 0.30 ml.; 4×10^{-3} *M* $AlCl_3$, 0.30 ml., and mitochondria suspension 0.2 ml. (talal volume 3.00 ml.)

succinoxidase and 10 per cent disappearance of -SH contents being observed. Methylbis (β-chloroethyl) and amine chlorovinyl-methyl-ketone, in particular the latter, were shown to be more reactive toward mitochondria than iodoacetamide, in so far as they attacked both enzymatic activity and -SH groups to the same extent.

Restoration of Succinoxidase Activity and -SH Content by GSH

The quite consistent parallelism between enzyme activity and residual -SH content as confirmed above urged the authors to examine whether or not GSH can restore -SH content of mitochondria as well as its enzymatic activity.

After the succinoxidase activity was inhibited to the extent of 80

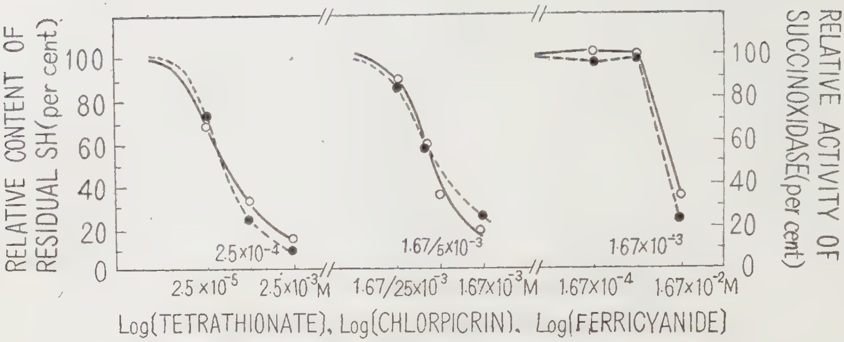

Fig. 2. Effect of oxidizing agents on succinoxidase and -SH content of mitochondria. —o—o— (Residual -SH content). - - -•- - -•- - - (Succinoxidase activity).

The experimental conditions are the same as described in Fig. 1.

TABLE I

Effect of Alkylating Agents on Succinoxidase and -SH Content of Mitochondria

Inhibitor	Final concentration	O_2 consumption per 10 minutes; its inhibition per cent in parenthesis		Residual -SH content; its reduced per cent ih parentheses	
	M	*μl.*	(*per cent*)	*μ.M*	(*per cent*)
Indo-acetamide	0	81.8		0.405	
	$1/6\times10^{-4}$	81.2	(0.7)	0.405	(0)
	$1/6\times10^{-3}$	78.0	(4.5)	—	(—)
	$1/6\times10^{-2}$	76.5	(6)	0.384	(5)
	$1/6\times10^{-1}$	64.5	(21)	0.360	(11)
Methyl-bis (β-chloroethyl) amine	0	59.5		0.390	
	2×10^{-3}	54.0	(9)	0.351	(10)
	5×10^{-3}	46.0	(22.7)	0.300	(23)
Chlorovinyl-methyl-ketone	0	31.0		0.330	
	$1/75\times10^{-2}$	16.5	(47)	0.210	(36·3)
	$1/15\times10^{-2}$	14.2	(54)	0.165	(50)
	$1/3\ \times10^{-2}$	12.0	(61.5)	0.135	(59.1)

All experimental conditions were the same as described in Fig. 1.

to 90 per cent by mercaptide-forming agents such as PCMB, $HgCl_2$ and $CuSO_4$ or oxidizing agents including tetrathionate and chloropicrin,

reactivation was attempted by the addition of excess of GSH. As shown in Fig. 3, the enzyme was reactivated considerably by GSH, its -SH content being simultaneously restored to the same extent in each case. The facts that GSH itself had no effect on succinoxidase activity and -SH content of mitochondria without inhibitors had been confirmed previously (*3*). In the reactivation by GSH in the case of tetrathionate, ratio of [GSH]: [Inhibitor] as low as 2.5:1 served quite satisfactory. The reversibility of chloropicrin inhibition further furnishes evidence for its oxidizing nature.

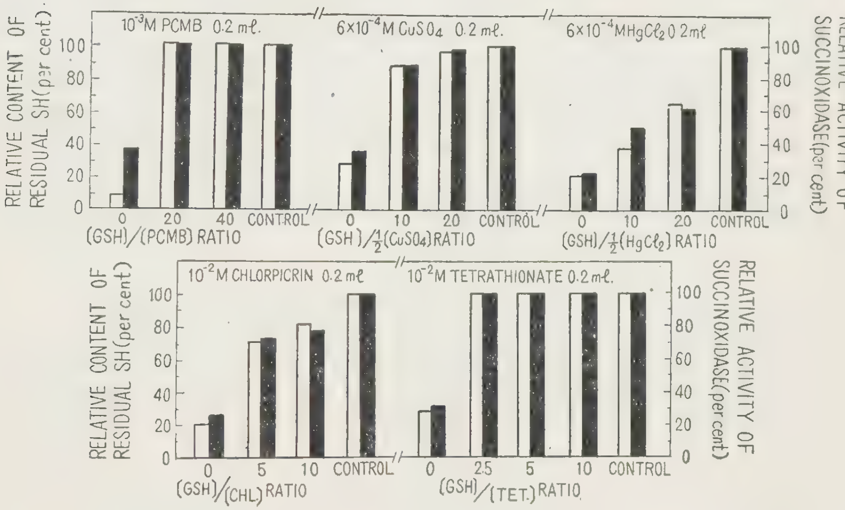

FIG. 3. Restoration of succinoxidase activity and -SH content by GSH
Black columns: residual -SH content in per cent.
White colums: succinoxidase activity in per cent.

Mitochondria were brought in contact with inhibitors for 10 minutes in reaction mixture of succinoxidase assay described in Fig. 1, then GSH was added at several ratios of GSH / Inhibitor, which are indicated under each figure. The ratios are expressed in term of normal concentration. Other experimental conditions were the same as in Fig. 1.

The Effect of Mercaptide-forming Agents on α-Ketoglutaric Oxidase Activity and -SH Content

Still (*14*) has assumed that α-ketoglutaric oxidase is also coherent in mitochondria. It is interesting, therefore, whether this enzyme shows similar behavior toward thiol-reagents as succinoxidase. The results

are shown in Fig. 4. In agreement with the expectation -SH content and enzyme activity diminished simultaneously by the action of three typical inhibitors, PCMB, $HgCl_2$ and $CuSO_4$.

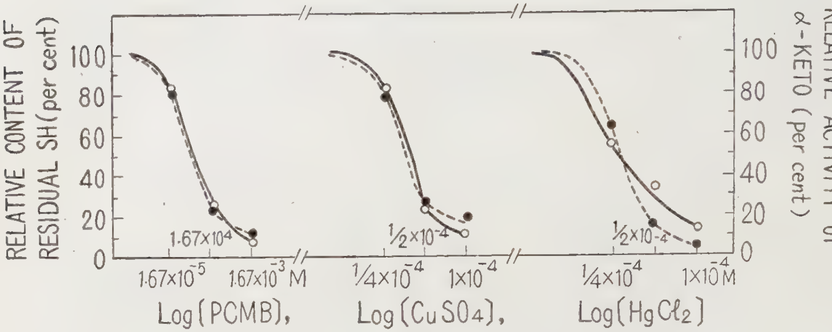

Fig. 4. Effect of mercaptide-forming agents on α-ketoglutaric oxidase activity and -SH content of mitochondria.

——○——○—— Residual -SH content.

- - -●- - -●- - - α-Ketoglutaric oxidase activity.

The reaction mixture of enzyme assay: water or solutions of inhibitors in different concentrations, 0.50 ml.; 0.1 *M* phosphate buffer (pH 7.4), 0.30 ml.; 1×10^{-4} *M* cytochrome c, 0.30 ml.; 0.05 *M* $MgCl_2$, 0.30 ml.; 0.15 *M* KCl, 0.80 ml.; 0.01 *M* ADP, 0.30 ml.; 0.10 *M* α-ketoglutarate, 0.30 ml.; and mitochondria suspension, 0.20 ml. (total volume, 3.00 ml.).

DISCUSSION

The first attempt to elucidate the relation between the activity of the enzyme and its -SH groups was made by Hellerman (*15*), who, by gradual destruction of the -SH groups with PCMB, found that crystalline urease contained about 5 moles of cysteine per unit of protein (213,000 g.), one of which being destroyed without effect on enzymatic activity, and the remaining four being quite essential for the activity. Barron *et al.* (*16*) applying the same method as Hellerman concluded that 93 per cent of the -SH groups of phosphoglyceraldehyde dehydrogenase was responsible for its activity. Singer *et al.* (*17*) treated a native crystalline β-amylase with *o*-iodosobenzoate until the inhibition of enzyme activity was nearly complete. At that time, however, only 25 per cent of the total -SH groups in the denatured protein (by Duponol PC) was oxidized.

On the basis of the experimental results already mentioned, it is obvious that in typical cases (including chlorovinyl-methyl-ketone) the parallelism between enzyme activity and -SH content was of satisfactory degree. This close agreement observed by the authors may indicate that in those experiments the -SH reagents had combined specifically with the enzyme and no nonspecific side-reactions occurred owing to the remarkably low final concentrations of the inhibitors. The inconsistency observed in the case of β-amylase by Singer (*17*) might be accounted for, at least partially, by the situation that the concentration of the inhibitors applied was relatively high.

Regarding inhibitory action of heavy metals, it must be pointed out that some heavy metals combine not only with -SH groups, but also with some carboxyl or amino groups of protein or amino acids, as shown by Haarman (*18*) as well as Borsook (*19*), Klotz (*20*), so that it is difficult to know what protion of heavy metal is used up to attack -SH group of the enzyme. But from the quantitative data of the inhibition experiments (Table II), it might be demonstrable that when

TABLE II

The Effects of Mercaptide-forming Agents on Succinoxidase Activity and -SH Content

Inhibitor	Inhibitor added		Residual -SH group found		*Residual inhibitor	O_2 consumption	
		μM	μM	*per cent*	μM	*µl./minutes*	*per cent*
PCMB	——	0	0.306	100	——	87.5	100
	10^{-2} *M* 0.5 ml.	5.0	0.045	14.2	4.749	13.9	15.9
	10^{-3} *M* 0.5 ml.	0.5	0.128	41.8	0.320	15.4	17.6
	10^{-4} *M* 0.5 ml.	0.05	0.255	83.0	0.00	72.4	83.0
	10^{-5} *M* 0.5 ml.	0.005	0.306	100.	0.005	87.5	100.
$HgCl_2$	——	0	0.330	100	——	76.5	100
	6×10^{-4} *M* 0.5 ml.	0.30	0.057	17.2	0.164	6.7	8.8
	$6/2\times10^{-4}$ *M* 0.5 ml.	0.15	0.081	24.5	0.025	11.3	14.8
	$6/4\times10^{-4}$ *M* 0.5 ml.	0.075	0.180	54.5	0.000	40.3	53.0
	$6/8\times10^{-4}$ *M* 0.5 ml.	0.0375	0.255	77.2	0.000	69.0	90.2
	$6/16\times10^{-4}$ *M* 0.5 ml.	0.0188	0.288	87.2	−0.003	70.0	91.5
$CuSO_4$	——	0	0.350	100	——	86.0	100.
	6×10^{-4} *M* 0.5 ml.	0.30	0.040	11.6	0.145	6.3	7.3
	$6/2\times10^{-4}$ *M* 0.5 ml.	0.15	0.230	65.7	0.120	60.0	70.
	$6/4\times10^{-4}$ *M* 0.5 ml.	0.075	0.300	85.5	0.050	74.0	86.
	$6/8\times10^{-4}$ *M* 0.5 ml.	0.0375	0.325	93.0	0.255	82.0	95.

Basic experimental condition for the data given in Fig. I.

* [Residual inhibitor]=[Inhibitor added, μM]—[-SH diminished, μM].

inhibitor is in "sufficently low" concentrations, the possiblity of its combination with any groups other than -SH would be negligibly small as in typical cases of PCMB and $HgCl_2$.

SUMMARY

1. In an attempt to follow out the correlation between the -SH contents and enzymatic activities studies were make on the effect of three main -SH reagents upon succinoxidase and α-ketoglutaric oxidase of isolated rat liver mitochondria as well as the reactivation experiments with GSH.

2. Mercaptide-forming agents such as PCMB, $HgCl_2$ and $CuSO_4$ diminished the -SH content in exact agreement with the inhibition of the enzyme activities of both types (Figs. 1, 4). The addition of GSH restored not solely the succinoxidase activity, but also proportionately the -SH content of the protein.

3. Oxidizing agents such as tetrathionate and chloropicrin caused a reversible inhibition of succinoxidase activity, -SH groups disappearing at equal rate with enzyme activity, while both were restored to the same extent by the effect of GSH.

4. Zn^{++}, Cd^{++}, iodoacetamide and ferricyanide showed poor inhibition of enzyme activity and low affinity toward -SH groups of the isolated mitochondria (Figs. 1 and 2, Table I). Chlorovinyl-methyl-ketone showed relatively high affinity.

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ENZYMATIC TRANSFER OF β-D-GALACTOSE

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In previous communications (*1*, *2*) it has been shown that the enzymatic hydrolysis of aryl-β-D-glucoside in the presence of a small amount of various kinds of alcohol is accompanied by the transfer of a part of β-D-glucose residue to alcohol, resulting in the formation of corresponding alkyl-β-D-glucoside. Such an enzymatic synthesis of alkyl-β-D-glucoside starting from aryl-β-D-glucoside and alcohol cannot be regarded as a synthesis in an ordinary sense, since in the absence of aryl-β-D-glucoside no alkyl-β-D-glucoside has been formed enzymatically from glucose and alcohol under otherwise analogous conditions of the reaction (*2*).

On the basis of the findings that the transfer activity was invariably linked with the hydrolyzing activity of β-glucosidase preparations of apricot emulsin as well as of purified preparations from molds such as *Aspergillus* and *Penicillium*, we have been led to conclude that the transfer activity of these enzyme preparations might be attributed to the action of β-glucosidase itself which has thus far been considered as a hydrolyzing enzyme.

In view of these results it appears of interest to examine whether or not such transfer action may also be found in other glycosidases. The present paper describes the experiments with β-D-galactosidase.

EXPERIMENTAL

As galactose donor (substrate) served *p*-nitrophenol-β-D-galactoside, and as galactose acceptor methanol and ethanol.

Enzymatic transfer reactions were carried out in the reaction mixture of the following composition:

0.048 *M* *p*-Nitrophenol-β-D-galactoside	2.0 ml.;
0.2 *M* Acetate buffer, pH 4.8	2.0 ml.;
5 *M* Alcohol	2.0 ml.;
Enzyme solution	4.0 ml.

Incubation temperature, 30°.

Controls containing 2.0 ml. water in place of alcohol were run to follow the course of hydrolysis without galactose transfer.

At varying time intervals 1.0 ml. aliquots were withdrawn and analyzed for liberated *p*-nitrophenol colorimetrically and galactose reductometrically. The extent of galactose transfer from *p*-nitrophenol-β-D-galactoside to alcohol is given by

$$\frac{[p\text{-Nitrophenol liberated}]-[\text{Galactose liberated}]}{[p\text{-Nitrophenol liberated}]}\times 100.$$

Experiments with Apricot Emulsin—Enzyme preparation was obtained according to the procedure of B. Helferich and associates (*3*). The activity approximated to that of "Rohferment" of Helferich.

TABLE I

Enzymatic Transfer of β-D-Galatose from p-Nitrophenol-β-D-galactoside to Alcohol by β-D-Galactosidase of Apricot Emulsin

Acceptor (alcohol)	Incubation time	*p*-Nitrophenol liberated	Galactose liberated	Galactose transferred to alcohol
	minutes	*per cent*	*per cent*	*per cent*
Methanol	25	25	21.2	14
	50	35	31.4	13
	95	50	40.4	19
Ethanol	25	22	18.0	18
	50	32	27.4	14
	95	45	35.7	21
Control (Water)	25	30	28.5	
	50	40	40.2	
	95	55	54.8	

The data listed in Table I indicate that while in the absence of alcohol the enzymatic cleavage of *p*-nitrophenol-β-D-galactoside resulted in the liberation of *p*-nitrophenol and galactose in equimolecular ratio, the presence of alcohol led to the liberation of galactose always smaller in amount than that of *p*-nitrophenol. This deficit of free galactose can be explained as having been transferred to alcohol.

The actual enzymatic transfer of β-D-galactose residue has been conclusively proved by the isolation from the reaction mixture of alkyl-β-D-galactoside. This experiment was performed with *o*-cresol-β-D-galactoside as donor and methanol as acceptor of β-D-galactose. Such a combination of donor and acceptor is particularly suited for methyl-β-D-galactoside to accumulate in the reaction mixture, as the rate of hydrolysis by apricot emulsin of this substrate is considerably large compared with that of the transfer product.

1.5 g. of *o*-cresol-β-D-galactoside were dissolved in a mixture of 70 ml. of 5 per cent methanol, 20 ml. of 0.2 *M* acetate buffer of pH 4.8 and 30 ml. of 0.5 per cent emulsin. Following incubation at 30° for 60 minutes the reaction mixture was heated to boiling and the precipitate formed was filtered off. The clear filtrate was evaporated to about 5 ml., to which was added 1 ml. of α-methylphenylhydrazine dissolved in 30 ml. of 50 per cent ethanol and the mixture was allowed to stand overnight at room temperature. The heavy precipitate of galactose-α-methylphenylhydrazone was filtered and washed with 50 per cent ethanol and water. The combined filtrate was freed from the excess of methyl-phenylhydrazine by shaking with benzaldehyde. The precipitated hydrazone was filtered off and the filtrate was concentrated to a thick syrup and exhaustively extracted with ether to remove benzaldehyde. The residue was taken up in 15 ml. of absolute ethanol, filtered and evaporated under

TABLE II

Fractionation of Apricot Emulsin with Respect to the Transferring and Hydrolyzing Activity

To 10 ml. of 0.5% emulsin (pH 4.5) was added with stirring 1 ml. of suspension of alumina gel B (40 mg. dry wt.) (*4*) and after occasional shaking for 30 minutes the mixture was centrifuged. The supernatant was diluted 5 folds with water for enzyme assay. From the adsorbate enzyme was eluted with 15 ml. of 0.2% diammonium phosphate. Following neutralization with dilute acetic acid the solution was made up to 20 ml.

Fraction	Acceptor	Incubation time	*p*-Nitrophenol liberated	Galactose liberated	Galactose transferred
		minutes	*per cent*	*per cent*	*per cent*
Eluate	Methanol	15	20	15.2	24
		30	32	23.6	27
		45	40	30.1	25
		90	58	44.0	24
	Water	15	18	17.0	
		30	30	30.1	
		45	38	37.4	
		90	58	56.7	
Supernatant	Methanol	20	28	22.8	19
		50	50	36.6	27
		80	70	49.2	30
	Water	20	28	26.6	
		50	50	50.1	
		80	70	69.1	

reduced pressure. The resulting syrup was dissolved in 5 ml. of hot ethanol and filtered. On keeping in a refrigerator the solution deposited crystals, which were filtered and recrystallized from ethanol. Thus 0.3 g. of the crystal with a melting point of 173° were obtained. Mixed melting point with an authentic specimen of methyl-β-D-galactoside (m.p. 174°) showed no depression.

The relatively strong transfer activity of the emulsin preparation itself suggests that the transfer action might be catalyzed by the ordinary β-D-galactosidase. In support of this concept the fractionation of the enzyme preparation has shown that the transfer activity is linked with the hydrolyzing one. The results are presented in Table II.

The enzymatic formation of methyl-β-galactoside from aryl-β-galactoside and methanol cannot be considered as due to a reverse reaction of the hydrolysis of methyl-β-galactoside, since, as Table III illustrates, no indication of the synthesis of methyl-β-galactoside in a dilute solution of free galactose (0.0096 M) and methanol (7.38 M) could have been detected.

TABLE III

Action of Apricot Emulsin on Dilute Solution of Galactose and Methanol

Final concentration: 0.0096 M D-galactose, 0.04 M acetate buffer (pH 4.8), 7.38 M methanol. Total volume: 10 ml., containing 50 mg. of emulsin.

Incubation time	Free galactose mg./10 ml.
minutes	
0	18.2
60	18.5
120	18.2
240	18.8

Experiments with the Enzyme Preparation from Leaves of Sambucus Sieboldiana (Elder)—Enzyme solution: Green leaves of elder tree which had been collected at the middle of June were air dried and finely pulverized. The powder was extracted with 5 times its weight of 0.05 M ammonia and the enzyme solution was separated by centrifugation. It was purified by deproteinization at pH 4.5 with 0.2 N acetic acid followed by dialysis. The hydrolyzing and transferring activities are indicated in Table IV.

These results are, as a whole, similar to those obtained with apricot emulsin (Table I). Namely the presence of alcohol has led to the formation of alkylgalactoside. In a separate run with 1.5 g. of p-nitrophenol-β-galactoside as donor and methanol as acceptor, crystals

TABLE IV

Enzymatic Transfer of β-D-Galactose from p-Nitrophenol-β-D-galactoside to Alcohol by an Enzyme from Elder Leaves

Acceptor	Incubation time	*p*-Nitrophenol liberated	Galactose liberated	Galactose transferred
	minutes	*per cent*	*per cent*	*per cent*
Methanol	90	28	12.5	56
	180	40	18.0	55
	270	57	22.3	61
	360	70	27.4	61
Ethanol	90	30	13.9	54
	180	40	18.0	55
	270	60	22.3	63
	360	70	27.4	61
Control (Water)	90	20	20.3	
	180	30	31.5	
	270	40	40.2	
	360	50	49.8	

of methyl-β-D-galactoside (m.p. 172°) were obtained.

DISCUSSION

The enzymatic transfer of β-D-galactose residue from aryl-β-D-galactoside to alcohol has been demonstrated in enzyme preparations of apricot emulsin and of elder leaves. As with the transfer of β-D-glucose the β configuration of D-galactose remained unchanged in the course of the transfer from phenol to alcohol. This fact might suggest the primary enzymatic activation of β-D-glucosyl or β-D-galactosyl group of the arylglycoside. The assumption of the primary hydrolysis of the glycoside bond followed by binding of the liberated nascent sugar with alcohol, as suggested by Rabaté (*5*), appears unlikely. Thus, as stated in a previous communication, (*2*), both water and alcohol act respectively as an acceptor for the sugar residue and in case of transfer to take place, the enzymatically activated sugar residue is dislocated from the substrate molecule and pass on, without intervention of hydrolysis, directly to alcohol.

The association of hydrolytic and transferring activity of apricot emulsin, as shown in the fractionation by adsorption and elution (Table II), may be taken to indicate that no particular enzyme other than ordinary β-D-galactosidase might be required for the transfer of β-D-galactose residue.

A comparison of the results obtained with apricot emulsin on the one hand (Table I) and those with the leaf enzyme on the other (Table IV) reveals that the transfer percentage by leaf enzyme is higher than that by apricot emulsin when methanol or ethanol is used as acceptor. This phenomenon may be accounted for as due to the difference in the relative acceptor specificity of β-D-galactosidases from different sources. Presumably there might exist glycosidases which perform exclusively hydrolysis without transfer activity even in the presence of the alcohol. Such enzymes must have strong affinity for water with no appreciable one for alcohol. Conversely enzymes with exclusive transfer activity such as levan and dextran sucrase may also occur. Between these two extremes, namely typical hyrdolase and transferase without hydrolytic activity, the existence of enzymes possessing both hydrolyzing and transferring activities in varying proportion depending on their source can be anticipated.

SUMMARY

1. By the action of enzymes of apricot emulsin and of green leaves of elder tree (*Sambucus Sieboldiana*) β-D-galactose residue of aryl-β-D-galactoside is transferred to alcohol forming alkyl-β-D-galactoside.

2. The association of hydrolyzing and trasferring activities of apricot emulsin has been considered as suggesting the identity of hydrolase and transferase, where water and alcohol act respectively as the acceptor of β-D-galactose residue.

3. The possibility of the existence of a variety of β-galactosidases with different affinities toward water and alcohols has been discussed.

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BILE ACIDS AND STEROIDS I.

STUDIES ON HOG BILE ACIDS (PART 1). NEW SYNTHETIC ROUTE OF METHYL Δ^4-3-KETOCHOLENATE FROM α-HYODESOXYCHOLIC ACIDS

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Hoehn *et al.* (*1*) has demonstrated that methyl α-hyodesoxycholate diacetate can be converted by partial saponification followed by chromic acid oxidation into 3-keto-6-α-hydroxycholanic acid. Chromic acid effects preferential oxidation at C_6 at a low temperature, but both hydroxyls at C_3 and C_6 are attacked at an ordinary tempetature. In hog bile, 3-hydroxy-6-keto-allocholanic acid always coexists with α- and β-hyodesoxycholic acids, and it is an interesting fact that hydroxyl group at C_6 is oxidized more easily than that at C_3 under such a reaction condition.

On the other hand, 3-keto derivatives are synthesized by Oppenauer oxidation of α-hyodesoxycholic acid (*2*, *3*), but the products are generally contaminated with by-products and the yields are small, or there are some difficulties in treatment.

According to Barton (*4*, *5*, *6*, *7*), and C. W. Shoppee, *et al.* (*8*), both 3-α- and 6-α-hydroxy groups are equatorial (*cf.* Fig. 1). Reexamination of the partial saponification of diacetate failed to yield the expected 3-hydroxy-6-acetoxy compound as described, but gave a 3,6-dihydroxy derivative.

The present paper describes the oxidation reaction of α-hyodesoxycholic acid with N-bromosuccinimide (NBS). α-Hyodesoxycholic acid was oxidized selectively at C_3 with NBS, and 3-keto-6-α-hydroxycholanic acid (**IIa**), m.p. 198–200° (corr.), $[\alpha]_D^{23}+13.7$ (methanol), ultraviolet spectrum maximum at 280 mμ (see Fig. 2), was obtained in a good yield. The best results were obtained with 1.1 to 1.3 equivalents of NBS in hydrated acetone, but a larger excess was found to give a bad effect. Both methyl ester (**IIb**) and its ester acetate of 3-keto-6-α-hydroxycholanic acid could not be obtained in crystalline form. This acid and its derivatives gave positive Jaffé and Zimmermann color tests.

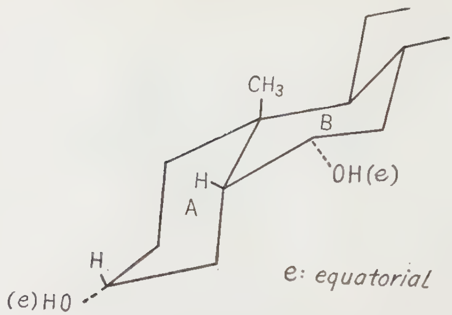
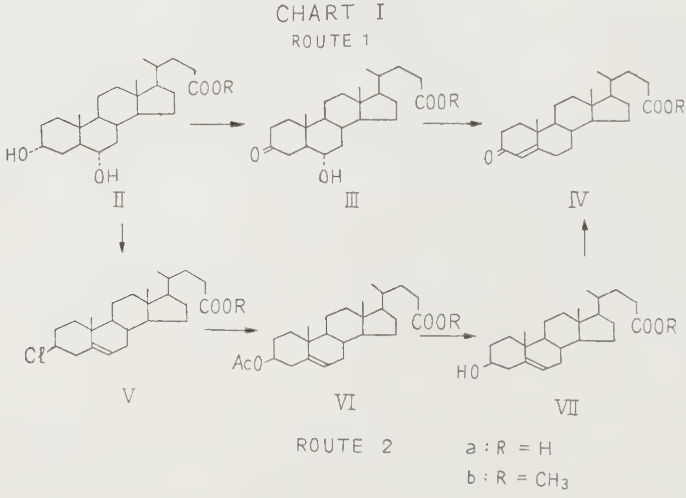
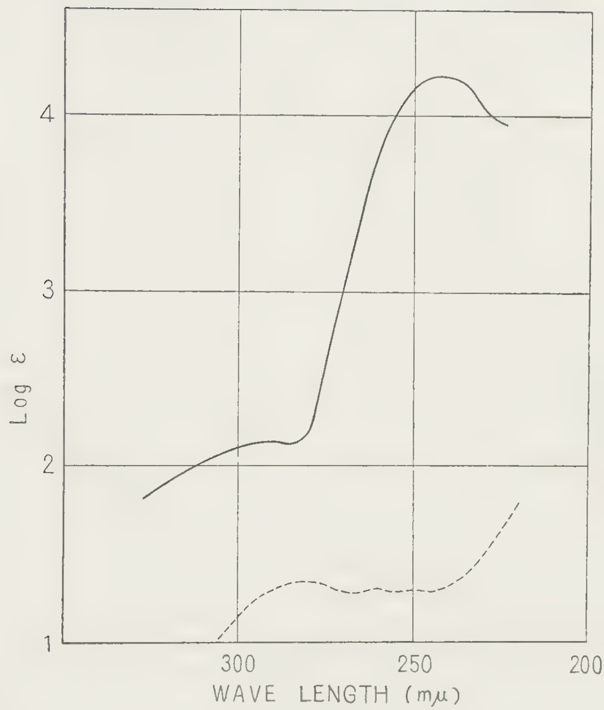


FIG. 1. Partial conformation of α-hyodesoxycholic acic by D.H.R. Barton *et al.*

This acid (**IIa**) showed marked melting point depression when admixed with the starting material, α-hyodesoxycholic acid, 3-hydroxy-6-ketoallocholanic acid, or 3,6-diketoallocholanic acid.



Oxidation of pure 3-keto-6-α-hydroxycholanic acid with 1.1 equivalents of NBS under the same conditions resulted in the recovery of the unchanged starting material. Also, 3-α-hydroxy-6-ketoallocholanic acid was not affected under the similar oxidation conditions. This reagent, NBS, thus, does not attack α-hyodesoxycholic acid at C_6, but attacks C_3 preferentially, and leaves C_6 untouched.



Ultraviolet absorption spectra in 94% ethylalcohol.
—— Me Δ^4-3-Ketocholenate
---- 3-Keto 6-hydroxycholanic acid

Methyl 3-keto-6-α-hydroxycholanate was dehydrated on treatment with phosphorous oxychloride in pyridine with the migration of one double bond and gave methyl Δ^4-3-ketocholenate (**IIIb**) in a comparatively good yield (37.5 per cent) (about the same amount as in the literature (*9*)).

Of course, dehydration through tosylate (*2*) affords better yields in all such dehydration reactions, but phosphorous oxychloride and pyridine were used in the present experiments in order to study the relationship between steric configuration of the hydroxyl group and the ease of dehydration.

The dehydration product (**IIIb**) showed melting point at 124.5–125.5°, $[\alpha]_D^{15}$ +66.13° (methanol), and ultraviolet spectrum maximum at 241 mμ (log ε = 4.2285) (*cf.* Fig. 2), E_{2410} = 16.935 (16.751). Saponification by

methanolic potassium hydroxide afforded the acid with melting point at 185–187°. These are identical with the data reported in the literature.

This compound was also synthesized by the following route (*10*) and its structure was confirmed. Methyl α-hyodesoxycholate was converted to methyl 3-β-chloro-Δ^5-cholenate with phosphorous oxychloride in pyridine, dehalogenation of this chloro compound followed by hydrolysis, gave 3-β-hydroxy-Δ^5-cholenic acid (**VI**), and by Oppenauer oxidation it furnished methyl Δ^4-3-ketocholenate (m.p. 124–125°), identical with the above-mentioned specimen.

EXPERIMENTAL*

ROUTE 1

Oxidation with N-Bromosuccinimide—6 g. of methyl α-hyodesoxycholate** were dissolved in 180 ml. of acetone, 25 ml. of water and 3 g. of N-bromosuccinimide were added, and the reaction mixture was allowed to stand for 4 days at room temperature. The solvent was removed under a reduced pressure and extracted with ether. The organic solvent layer was washed and extracted with dil. sodium carbonate solution. The crude acid obtained from this alkaline solution was recrystallized from ethyl acetate, it melted at 177–187°. Yield, 600 mg.

Further recrystallization from acetone gave *ca.* 500 mg. of nearly pure 3-keto-6-hydroxychlolanic acid (m.p. 195–6°). The neutral fraction was purified by chromatography on alumina. Elutions with a petroleum ether-ether mixture (200 ml.) and with ether alone (*ca.* 500 ml.) gave *ca.* 5.3 g. of oily methyl 3-keto-6-hydroxycholanate, which gave 2.7 g. of the free acid (m.p. 193–8°). The total yield of this 3-keto acid was 3.2 g. (53 per cent). Further elution with a ether-benzene mixture (400 ml.) gave *ca.* 280 mg. of the starting material. Relation between the reaction time and the percentage of the recovered starting material is shown in Fig. 3.

FIG. 3. Relationship between the reaction time and the percentage of recovered material on the NBS oxidation (16-18°)

* All melting points reported are uncorrected.

** Methyl α-hyodesoxycholate (m.p. 100–102°).

The melting point differs widely from that reported by the American researchers.

The pure acid melted at 198–200° (*corr.*) from acetone. $[\alpha]_D^{23}+13.7$ (methanol, $l=1$ dm.); 18.973 mg. in 2 ml. methanol, $\alpha=+0.13$.

Analysis: Calcd. for $C_{24}H_{38}O_4$, C 73.80%; H 9.81%
Found, C 73.42%; H 9.48%

Ultraviolet spectrum (see, Fig. 2.): a maximum at 280 mμ ($\log \varepsilon = 1.354$).

Another Oxidation Experiment—Oxidation of α-hyodesoxycholic acid (2 g.) in the same way with 1.33, 1.5 and 2.0 equivalents of NBS gave *ca.* 145 g. of m.p. 185–190°, *ca.* 1.83 g. of m.p. 181–3° and *ca.* 0.9 g. of m.p. 188–190°, respectively; and all the products were contaminated with the starting material. In the latter case, the product showed positive Beilstein test and was troublesome in treatment. Attempted oxidation in aqueous bicarbonate solution (*11*) or in dioxane solution at 25° was unsuccessful.

Attempted Oxidation of 3-Keto-6-α-Hydroxycholanic Acid with N-Bromosuccinimide—1 g. of 3-keto-6-α-hydroxycholanic acid was treated in 50 ml. of acetone and 5 ml. of water with *ca.* 0.5 g. of NBS. The solution was allowed to stand for 16 hours (the usual color changes occurred) and treated exactly as above, from which the recovered 3-keto-6-α-hydroxycholanic acid was the only product.

Treatment of 3-Hydroxy-6-ketoallocholanic Acid with N-Bromosuccinimide—The same oxidation reaction as above was attempted on 3-hydroxy-6-ketoallocholanic acid, but it proved to give no effect.

Methyl Δ^4-3-Ketocholenate—800 mg. of 3-keto-6-α-hydroxycholanic acid (m.p. 198–200°) were esterified with diazomethane, dried, and dissolved in pyridine (20 ml.). Approximately 5 ml. of freshly redistilled phosphorus oxychloride were added and the mixture was allowed to stand for 4 days at a room temperature (18°). After decomposition with dilute hydrochloric acid solution and ice-water, it was extracted with an ether-ethyl acetate mixture (3:1) and washed consecutively with dilute hydrochloric acid solution, dilute sodium carbonate solution and water. After drying over sodium slufate, the residue was purified by chromatography on alumina. The separated oily substance which crystallized gradually when allowed to stand or by treatment of its acetone solution with a little water, was recrystallized from petroleum-ether (b.p. 40–60°) and it melted at 124.5–125.5°. Yield, *ca.* 350 mg. (37.5 per cent). $[\alpha]_D^{15}+66.13$ (methanol, $l=1$ dm.); 19.780 mg. in 2 ml. methanol, $\alpha=+0.654$; ultraviolet spectrum (see, Fig. 2), $E_{2410}=16.935$ (16.751). The compound gave no depression of the melting point on admixture with an authentic sample (*Route 2*).

Analysis: Calcd. for $C_{25}H_{38}O_3$, C 77.67%; H 9.91%
Found, C 77.30%; H 9.54%

When the crude oily methyl 3-keto-6-α-hydroxycholanate obtained by the chromatographic separation of the ester of the acid of m.p. 190–191°, was used, the yield of methyl Δ^4-3-ketocholenate was only 31.5 per cent.

Δ^4-3-Ketocholenic Acid (**IIIa**)—300 mg. of methyl Δ^4-3-ketocholenate were dissolved in 50 ml. of 0.5 *N* aqueous sodium hydroxide solution and 50 ml. of methanol. The mixture was refluxed for 3 to 4 hours on a water-bath. This mixture was cooled, filtered, and concentrated under a reduced pressure, then acidified with dilute hydrochloric acid solution. The precipitate thereby formed was filtered, washed with water,

and dried at 70° under a reduced pressure. Recrystallization from ethyl acetate gave 200 mg. of plates, m.p. 175–180° (*ca.* 60 per cent.) The pure acid melted at 185–187°.

Analysis: Calcd. for $C_{24}H_{35}$-O_3, C 77.58%; H 9.49%
Found, C 77.10%; H 9.47%

ROUTE 2

Methyl Δ^4-3-Ketocholenate by the Oppenauer Oxidation—200 mg. of 3-β-hydroxy-Δ^5-cholenic acid (*7*) were esterified with diazomethane and the ester was dissolved in 5 g. of dry accetone. To this were added 0.5 g. of aluminium isopropoxide in 15 ml. of absolute benzene. After working up in the usual manner, the yellow oil obtained was chromatographically purified and *ca.* 100 mg. of a product (m.p. 118–120°) were obtained. Recrystallized from petroleum ether (b.p. 40–60°), it melted at 124–125°. The melting point was not depressed on admixture with the product obtained by the *Route 1.*

Analysis: Calcd. for $C_{25}H_{38}O_3$, C 77.67%; H 9.91%
Found, C 77.33%; H 9.45%

SUMMARY

An improved method for the conversion of α-hyodesoxycholic acid into 3-keto-6-α-hydroxycholanic acid has been found in the oxidation of the free acid or ester at C_3 with N-bromosuccinimide in hydrated acetone solution in a quantitative yield. The oxidation agent acts selectively and the hydroxyl group at C_6 remains unattacked. And 3-keto-6-hydroxy acid is converted easily into Δ^4-3-keto acid by the usual method.

We express our sincere gratitude to Prof. Dr. Yamasaki, Tottori University, Yonago, who generously sent us the sample of 3-hydroxy-6-ketoallocholanic acid. The author thanks Mr. T. Ieki and Mr. K. Miyahara for microanalyses, and Messrs T. Kubota and Y. Matsui for the determination of ultraviolet absorption spectra.

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THE STRUCTURE OF ADENYLTHIOMETHYLPENTOSE. I*

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Adenylthiomethylpentose (ATMP) is a characteristic sulfur-containing nucleoside, first isolated from yeast by Mandel and Dunham in 1912 (*1*) and then from oryzanin (*2*), brewer's yeast (*3*) and from impure cozymase (*4*). On hydrolysis it yields one mole adenine and one mole sugar which was found by Suzuki *et al.* (*2*) to contain sulfur. According to Nakahara *et al.* (*5*) adenylthiomethylpentose has the same effect as vitamin L_2, while vitamin L_1 is said to be anthranilic acid.

Falconer and Gulland (*6*) stated that the thiosugar is combined to the 9-position of the adenine base. The thiosugar has been the subject of several investigations. According to Suzuki, Ohdake and Mori (*1, 7*) the sugar gives besides Molisch test all the other reducing sugar tests, strong pentose reaction with Bial's reagent and also furfurol reaction, but gives neither the methylpentose reaction nor the Selivanoff's.

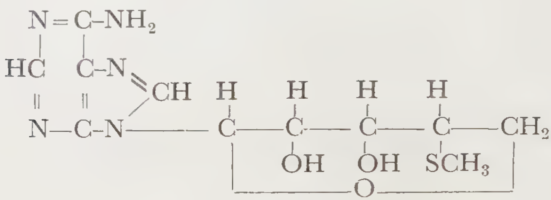
The sulfur can be detected only after fusion of thiosugar with metallic sodium. Suzuki *et al.* (7) assigned to the thiosugar the structure of 5-thiomethyl aldopentose and showed that the thiosugar could be reduced by means of sodium amalgam to the thiomethyl group containing polyalcohol (thiopentitol), having the formula, $C_6H_{14}SO_4$ (**I**).

$$\underset{\displaystyle OH}{CH_2}—\underset{\displaystyle OH}{CH}—\underset{\displaystyle OH}{CH}—\underset{\displaystyle OH}{CH}—CH_2—SCH_3 \qquad \textbf{(I)}$$

Levene and Sobotka (*8*) assigned to the thiosugar the structure of 3-or 4-thiomethyl ketopentose, based on its bromine oxidation. Suzuki *et al.* and Levene *et al.* showed that the thiomethyl group was retained during osazone formation, excluding position 1 and 2 of the thiosugar as the combining position for the thiomethyl group. A paper by Wendt (*9*), based on titration by the Willstätter-Schudel

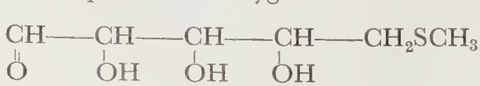
* Preliminarily reported in *Nature*, **165**, 767 (1950).

method, showed that the thiosugar was an aldose, and it was then demonstrated that, on decomposition with lead tetraacetate according to Criegee, the thiopentitol yielded 0.9 moles of formaldehyde, indicating that the 3-position of the thiosugar could not be the combining position for the thiomethylpentose. But this result proves only that the thiomethyl group cannot be in the 3-position, and offers no definite explanation for deciding whether the thiomethyl group is attached to the 4- or 5-position: that is, other structural formula, especially (**II**) can not be excluded. Hence the structure of ATMP has not been definitely established.



(**II**)

So the author made some experiments in this respect. When the ATMP was submitted to periodate oxidation, two oxygen atoms were used as indicated in Table I; one of which is definitely consumed by the thiomethyl group, because methionine used also one oxygen atom with periodate, leaving the amino acid portion unaffected. Phenylalanine tested as the control consumed no periodate (Table II). So there is used only one oxygen by the other portion of the sugar moiety like the ordinary nucleoside (adenosine, guanosine—Table II), and in the mother liquor no formic acid was detected. Thus the furanoside structure of this thiomethylpentose was proved. It was found that the thiosugar solution obtained by heating the ATMP with dilute sulfuric acid (normal solution) for one hour and a half in a sealed tube, required 1.9 moles of iodine in the Willstätter-Schudel titration (*10*) (Table III), indicating that both aldehyde and thio groups are almost simultaneously oxidized, for thiopentitol (**I**), which has no aldehyde group, also consumed 0.9 moles of iodine to form the sulfoxide. A newly prepared thiosugar solution took up four atoms of oxygen with periodate (Table IV), indicating that the thiosugar has the structure (**III**), for (**IV**) must require three oxygen atoms in all.



(**III**)

$$\begin{array}{ccccccccc} CH & - & CH & - & CH & - & CH & - & CH_2OH \\ \| & & | & & | & & | & & \\ O & & OH & & OH & & SCH_3 & & \end{array} \qquad \textbf{(IV)}$$

Thiopentitol (**I**) consumed exactly four atoms of oxygen with periodate (Tables V and VI), yielding 0.9 moles of formaldehyde (detected as formaldimedone (Table VII) or determined by the Ripper's sodium bisulfite method) (Table VIII) after distillation of the mother liquor, and two moles of formic acid (Table IX) titratable with alkali. These results indicate that the natural thiopentose must have the thiomethyl group in position 5, excluding the possibility of other positions.

By the above experiments it can be proved that the ATMP has the structure of adenyl-5-thiomethylfuranopentoside.

If the investigation by Raymond (*11*) stating that the osazone of thiomethylpentose was not identical with that of 5-thiomethylxylose, is correct, this thiosugar must belong to the ribose or arabinose type. The solution of this problem awaits further research.

EXPERIMENTAL

I. Periodate Oxidation (*12*):

a) *Potassium Metaperiodate*—The preparation of potassium metaperiodate was carried out according to the direction of Langlois (*13*).

b) 0.05 *M Potassium Periodate Solution*—The potassium periodate solution was prepared by dissolving 1.150 g. of potassium periodate in 30 ml. of *N* sulfuric acid by heating slightly and filled up to 100 ml. with water (2.00 ml. of this solution=8.00 ml. of 0.1 *N* sodium thiosulfate).

c) *Periodate Oxidation of ATMP and Methionine*—29.7 mg. of ATMP were added to 0.05 *M* potassium periodate solution and were allowed to stand for twelve hours at room temperature. After addition of *N* sulfuric acid and 20 per cent potassium iodide solution, the liberated iodine was titrated with 0.1 *N* sodium thiosulfate.

TABLE I

Periodate Oxdation of ATMP

Substance	0.05 *M* KIO_4 solution added	Ratio of moles	0.1 *N* sodium thiosulfate used	Oxidant consumed
mg.	*ml.*		*ml.*	*moles*
ATMP 29.7	2.00	1 : 1	4.10	—
" "	4.00	1 : 2	12.01	2
" "	6.00	1 : 3	20.01	2
" "	8.00	1 : 4	27.96	2
—	2.00	—	8.00	—

Though was added varying excess of oxidant the moles of oxidant used were all the same. In the mother liquor no formic acid was detected.

For the purpose to determine the effect of thiomethyl group upon the consumption of periodate the author performed an experiment which consisted of the following series (Table II).

TABLE II

Periodate Oxdation of Amino Acid and Nucleosides

Substances		0.05 M KIO_4 solution added	Ratio of moles	0.1 N Sodium thiosulfate used	Oxidant consumed
	mg	*ml.*		*ml.*	*moles*
Phenylalanine	8.25	2.00	1 : 2	8.0	0
Methionine	3.73	2.00	1 : 4	7.5	1.0
Adenosine	13.80	4.00	1 : 4	15.1	0.9
Guanosine	15.95	2.00	1 : 2	7.0	1.0

The result indicates that one mole of oxidant is definitely consumed by the thiomethyl group.

II. Determination of Thiosugar by Willstätter-Schudel's Method (10)—As thiosugar had not yet been prepared in crystalline form, the hydrolysate of ATMP was directly used as the sugar solution to be determined by Willstätter-Schudel's method. The thiosugar solution obtained by heating with N sulfuric acid for one hour and a half in a sealed tube now required 1.9 moles of iodine (Table III), while thiopentitol, which has no aldehyde group, consumed 0.9 moles of iodine when the thio-alcohol is heated with N sulfuric acid for one hour before the titration with sodium hypoiodite, and consumed one mole of iodine after treating with N sulfuric acid at room temperature. So the iodine which was consumed by the sugar moiety after the acid hydrolysis of ATMP is nearly one mole. So the thiosugar must be (regarded as) an aldose. Adenosine, which was hydrolyzed by heating with 0.1 N sulfuric acid, consumed only one mole of iodine (Table III).

III. Periodate Oxidation of Thiosugar—The thiosugar solution which was prepared as above by heating with N sulfuric acid for one hour and a half in a sealed tube, took up four moles of oxidant as seen in the following Table IV.

Adenosine consumed the theoretical moles of oxident after the hydrolysis with 0.1 N sulfuric acid for one hour, while the hydrolysis of ATMP seems more difficult and the theoretical consumption of oxidant was attained only after the hydrolysis with N sulfuric acid for one hour and a half. A series of pentoses which were tested for comparison also took up the theoretical amounts of oxidant as can be seen from the Table IV.

IV. Periodate Oxidation of Thiopentitol:

a) Periodate Consumption—To 4.55 mg. of thiopentitol was added 0.05 M potassium periodate solution and, after the mixture was allowed to stand for twenty-four hours with occasional shakings, N sulfuric acid and 20 per cent potassium iodide solution,

Table III

Determination of Thiosugar by Willstätter-Schudel Method

Substances		Time of hydrolysis	0.02 *N* iodine	0.1 *N* NaOH	0.02 *N* Sodium thiosulfate used.	Iodine consumed
	mg	*hours*	*ml.*	*ml.*	*ml.*	*moles*
ATMP	2.97	0.5	2.0	0.6	0.12	1.88
	"	1.0	"	"	0.11–0.09	1.89–1.91
	"	1.5	"	"	0.08–0.09	1.91–1.92
	"	2.0	"	"	0.19–0.17	1.81–1.83
Adenosine*	2.76	1.0	2.0	0.6	1.00	1.00
	"	2.0	"	"	0.95	1.05
Thiopentitol	4.55	—	2.0**	3.5	1.50**	1.0
	"	1.0	" **	"	1.55**	0.9

The hydrolyzate obtained by heating the test materials with 1 ml. of *N* sulfuric acid in a sealed tube, was neutralized with *N* sodium hydroxide, mixed with 0.02 *N* iodine and 0.1 *N* sodium hydroxide solution, and, after standing four hours, acidified slightly with 0.1 *N* sulfuric acid and titrated with 0.1 *N* sodium thiosulfate.

* Adenosine was hydrolyzed with 0.1 *N* sulfuric acid.

** 0.1 *N* iodine and 0.1 *N* sodium thiosulfate solution.

Table IV

Periodate Oxidation of Thiosugar

Substances		H_2SO_4 added		Time of hydrolysis	0.05 *M* KIO_4 added	0.1 *N* $Na_2S_2O_3$ used	Oxidant consumed
	mg.	*ml.*	*N*	*hours*	*ml.*	*ml.*	*moles*
Adenosine	6.9	1	0.1	0.5	2.0	6.80	2.4
"	"	1	0.1	1.0	2.0	6.05	3.9
ATMP	7.43	—	—	—	2.0	6.97	2.1
"	"	1	1	0.5	2.0	6.55	2.9
"	"	1	1	1.0	2.0	6.20	3.6
"	"	1	1	1.0	2.0	6.10	3.8
D-Riobose	9.5	—	—	—	4.0	12.10	3.9
2-Desoxy-D-ribose	6.7	—	—	—	4.0	13.87	2.1
L-Arabinose	7.5	—	—	—	4.0	12.10	3.9
D-Xylose	7.5	—	—	—	4.0	12.01	4.0

were added and the liberated iodine was titrated with 0.1 *N* sodium thiosulfate. Though was added varied excess of oxidant, the moles of oxidants consumed were the same in all cases. For comparison polyalcohols, such as adonitol, dulcitol and sorbitol, were submitted to the periodate oxidation and the theoretical consumption of the oxidant was seen in all cases as demonstrated in Table V.

TABLE V

Periodate Oxidation of Thiopentitol and Polyalcohol

Substances		0.05 M KIO_4 solution added	Ratio of moles	0.1 N Sodium thiosulfate used	Oxidant consumed
	mg.	*ml..*		*ml.*	*moles*
Thiopentitol	4.55	2.0	1 : 4	6.01	3.98
,,		3.0	1 : 6	10.01	3.98
,,		4.0	1 : 8	14.00	4.00
,,		5.0	1 : 10	18.05	3.9
Adonitol	7.6	4.0	1 : 4	12.02	3.98
Sorbitol	9.5	6.0	1 : 6	19.20	4.8
Dulcitol	9.1	6.0	1 : 6	19.00	5.0

b) *Periodate Oxidation without Using Sulfuric Acid*—As the addition of sulfuric acid to the periodate oxidation mixture makes it uneasy to detect formic acid, the addition of sulfuric acid was avoided when there was necessity to observe the formic acid formation. Without sulfuric acid, the periodate consumption of the test substances was the same as the cases in which it was used. The experiment was performed as follows: To 46.00 mg. of potassium periodate in a small amount of water were added the substances to be tested, and the mixture without adding sulfuric acid, was allowed to stand for twenty-four hours with occasional shakings and, after addition of N sulfuric acid and 20 per cent potassium iodide solution, the liberated iodine was titrated with 0.1 N sodium thiosulfate. The results are summarised in the following table. (Table VI).

TABLE VI

Periodate Oxidation without Using Sulfuric Acid

Substances		KIO_4 added	0.0 N Sodium thiosulfate used	Oxidant consumed
	mg.	*mg.*	*ml.*	*moles*
Adonitol	7.6	46.00	12.02	3.98
Thiopentitol	9.1	46.00	12.05	3.95
Rhamnose	9.1	46.00	12.03	3.97

V. Determination of Formaldehyde—The formaldehyde produced by the periodate oxidation was determined by the following two methods.

a) *Gravimetric Method*—The resulting solution following the periodate oxidation as described in *Paragraph IV, b*), was steam-distillated with the apparatus of Criegee (*14*) and to the distillate was added 10 per cent alcoholic solution of dimedone and heated on a boiling water-bath, acidified with acetic acid and cooled to room temperature. Formaldimedone (m.p. 188–9°) thus obtained was determined by gravimetric method (*15*).

TABLE VII

Determination of Formaldehyde by Gravimetric Method

Substances		KIO_4 added	Formaldimedone	Formaldehyde	
	mg.	*mg.*	*mg.*	*mg.*	*moles.*
Thiopentitol	9.1	46.00	13.158	1.355	0.98
Adonitol	7.6	46.00	26.532	2.733	1.87

b) *Ripper's Sodium Bisulfite Method* (*16*)—The mixture of potassium periodate and each test substance was allowed to stand for twenty-four hours with occasional shakings, neutralized with 0.1 *N* sodium hydroxide solution, and steam-distillated. To the distillate was added 10 ml. of the standard sodium bisulfite solution and the mixture was allowed to stand for four hours in a dark place, and after adding 0.01 *N* iodine, titrated with 0.01 *N* sodium thiosulfate.

Calculation: ml. of 0.01 *N* sodium thiosulfate$\times$0.3002/2=formaldehyde in mg.
ml. of 0.01 *N* sodium thiosulfate$\times$0.4403/2=acetoaldehyde in mg.

TABLE VIII

Determination of Aldehyde by Ripper's Method

Substances		KIO_4 added	Aldehyde calculated		0.1 *N* NaOH	0.01 *N* $Na_2S_2O_3$ used	Aldehyde	
	mg.	*mg.*		*mg.*	*ml.*	*ml.*	*mg.*	*moles*
Adonitol	1.52	9.2	HCHO	0.6	0.31	3.81	0.571	1.91
Rhamnose	3.64	18.4	CH_3CHO	0.88	0.82	2.90	0.638	0.72
Adonitol+	1.52+	27.6	HCHO	0.6	1.18	6.70	—	2.63
Rhamnose	3.64		CH_3CHO	0.88				
Formalin	0.60	—	HCHO	0.60	—	3.96	0.594	0.99
Thiopentitol	3.03	18.03	HCHO	—	0.34	3.16	4.75	0.95

From the above Tables VII and VIII it can be seen that all the substances tested yielded the theoretical moles of aldehyde.

VI. Determination of Formic Acid—The solution of the test substances, after oxidized

TABLE IX

Determination of Formic Acid

Substances		KIO_4	0.1 *N* NaOH used	Formic acid
	mg.	*mg.*	*ml.*	*moles*
Adonitol	7.6	46.0	1.51	3.02
Thiopentitol	9.1	46.0	1.02	2.04
Rhaminose	9.1	46.0	2.08	4.16
TAMP	14.8	60.0	0.0	0.0
Thiopentitol	9.1	60.0	1.03	2.06

with periodate without using sulfuric acid, was titrated with 0.1 *N* sodium hydroxide solution using methyl red as an indicator.

As can be seen from the above table (Table IX), all the substances tested, yielded the theoretical amount of formic acid with the exception of ATMP. The latter, which has only two adjacent hydroxyls, produced no formic acid on oxidation with periodate.

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ON THE CHEMICAL NATURE OF BLOOD COAGULATION ACCELERATORS CONTAINED IN THE BONE MARROW EXTRACT

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(Received for publication, June 22, 1953)

The mechanism of blood coagulation is enormously complex, and despite the efforts made by many investigators over a long period since Morawits introduced his theory in 1904, the problem still remains unsolved. In 1932 Saito (*1*) made bone marrow extract from long tubular bones of domestic fowls and distinctly recognized its blood coagulation accelerating action in animal experiment. He thought that the bone marrow has a function somewhat resembling to the glands secreting some hormones. Since then the bone marrow extract has been used in practical medicine as blood coagulant. But the chemical nature of its principle is still obscure.

The author separated two fractions having physiological action by paper chromatography from the extract of bone marrow (Medullan) which has a remarkable accelerating action on the blood coagulation and found that hypoxanthine, inosine and guanosine are contained in them and observed that pure samples of them all have blood caogulation accelerating action. Furtheremore, the author recognized that they are effective only *in vivo* and no activity was detected *in vitro*. However, the detailed data concerning the animal tests, which were carried out with pure hypoxanthine, inosine and guanosine, will be given in the following report (*2*).

EXPERIMENTAL

Material

A bone marrow preparation called " Medullan " was used, which was prepared from long tubular bones of domestic fowls; after removal of lipids with alcohol, acetone, and ether, extraction was made with physicological saline and then the extract was deproteininzed with

sulfosalicylic acid. The excess in sulfosalicylic acid was removed with calcium carbonate and filtered. This filtrate is no more than " Medullan," and 1 ml. of this showed a remarkable accelerating action on the blood coagulation, as shown in Table I, the shortening in clotting time being approximately one-third of the normal case.

A 40 ml.-portion of this preparation was concentrated to a small amount *in vacuo* at 50°, then evaporated to dryness in a vacuum desiccator over calcium chloride and treated repeatedly with alcohol for desaltation. The alcoholic solution was evaporated to approximately 0.2 ml.

Separation of Substances by Paper Chromatography

For the purpose of studying the relation between the substances contained in the bone marrow extract and the blood coagulation, the syrup-like material prepared as mentioned above was paper-chromatographed at room temperature with ascending method using water-saturated butanol and a filter paper strip No. 50 of Toyo Roshi (Japan).

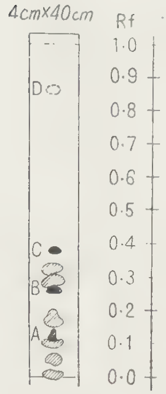
As shown in Fig. 1, three spots, A, B and C with different Rf values and one spot, D, having a light purple fluorescence were detected by the use of the ultraviolet light detector. At the same time, six spots, $N_1 \sim N_6$, showing a purple color, were detected with the use of ninhydrin indicating the existence of several amino acids. The Rf values of *Spots A*, *B*, *C*, and *D* are 0.13, 0.26, 0.38, and 0.86, respectively; the Rf values of these spots colorized with ninhydrin were 0.01, 0.05, 0.1, 0.16, 0.29, and 0.33 in order from N_1 to N_6.

Then, the same method was used only developing the test materials in a band-like form on a wide filter paper of 40 cm. $\times$ 40 cm. Thereafter the whole filter paper was cut in nine sections separately corresponding to the individual Rf values of the above spots as much as possible, and then each strip was cut slenderly at the width of 1 mm., put in separate flasks with 10 ml. of water which were shaken for 10 hours with an electrical shaker. This elute was filtered with suction, and 10 ml. of water were added again to the filter paper, shaken for 5 hours and filtered. Then these two filtrates were put together, and the whole elute was concentrated to 5 ml. and used for animal experiment. (see Fig. 2)

Effects of separated Fractions upon Blood Coagulation

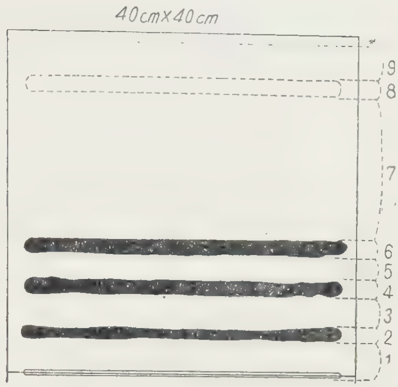
Animal experiment was performed as follows: With 12 rabbits as test animals, 1 ml. of each of the elutes obtained as described above was injected in the ear vein of rabbits weighing from 2.0 kg. to 2.5 kg.,

Fig. 1



: Ninhydrin positive Spots $N_1 \sim N_6$

Fig. 2



* No. of Section to be cut off for elution

and the time required for blood coagulation was measured every 30 minutes for 3 hours after the injection. The Sahli-Fonio method modified by Tanaka (*3*) was applied at 20°. When the same rabbits were to be used repeatedly, an interval of at least 7 days was laid and the following experiment was conducted after the blood coagulation time of the test animals returned to normal.

The result showed that the elutes from the A and B fractions distinctly accelerated the blood coagulation of healthy rabbits but the remaining seven fractions showed no activity. For the purpose of describing briefly, the results are summarized in the accompanying table. Among the nine fractions, the activities of the elutes from the A and B fractions which accelerate blood coagulation, and one (Table II) of the seven fractions that have no activity are shown here. Moreover, in regard to the A and B fractions, there are also demonstrated three cases of subcutaneous injection. (see Tables I∼VI)

Chemical Nature of Spots A, B and C

In order to identify the active substances contained in the A and B fractions and the substance contained in C fraction wich have no accelerating action, various investigations were carried out as follows:

Spot B—By the paper chromatography using *n*-butanol saturated with water as the developer, *Spot B* showed an Rf value of 0.26. However, there are several substances that have very close Rf values with

TABLE I

Blood Coagulation Time after Intravenous Injection of Medullan

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♀	2.3	18.0	15.5	11.0	11.0	12.5	14.5	17.5
2	♀	2.5	19.5	14.0	13.0	11.5	14.0	13.5	18.0
3	♂	2.5	19.5	15.5	13.5	12.0	14.0	14.0	18.5
Average			19.0	15.0	12.5	11.5	13.5	14.0	18.0

TABLE II

Blood Coagulation Time after Intravenous Injection of Elute of Spots N_1 and N_2

Rabbit No.	Sex	Body weight	Before injection	Time after injection imminutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♀	2.0	16.5	16.0	17.5	——	18.0	16.5	17.0
2	♂	2.3	16.5	16.5	16.0	17.0	16.5	15.0	17.0
3	♂	2.4	18.0	17.5	18.5	18.5	18.0	16.5	18.5
Average			17.0	16.7	17.3	17.7	17.5	16.0	17.5

TABLE III

Blood Coagulation Time after Intravenous Injection of Elute of Spot A

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♂	2.5	20.0	15.5	14.0	15.0	14.0	17.0	19.0
2	♂	2.5	16.5	13.5	13.5	11.5	12.5	——	15.0
3	♀	2.3	16.5	14.5	14.5	12.5	11.0	14.5	16.0
4	♀	2.0	18.5	18.0	14.5	——	11.5	——	19.0
5	♀	2.4	17.5	14.0	13.0	12.5	12.0	17.0	18.0
Average			18.0	15.1	13.9	13.0	12.2	16.2	17.3

TABLE IV

Blood Coagulation Time after Subcutaneous Injection of Elute of Spot A

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♀	2.3	19.0	19.0	14.5	14.0	12.5	13.5	13.5
2	♂	2.1	17.5	18.0	13.5	11.5	11.5	13.0	14.0
3	♀	2.4	19.0	17.5	13.0	12.0	13.5	12.5	13.0
Average			18.5	18.2	13.7	12.5	12.5	13.0	13.5

TABLE V

Blood Coagulation Time after Intravenous Injection of Elute of Spot B

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♂	2.5	19.5	17.5	15.0	15.0	14.5	16.5	21.0
2	♂	2.5	21.5	16.5	15.5	15.5	13.0	15.5	19.5
3	♀	2.3	180.	14.5	12.0	12.0	14.0	16.5	16.5
4	♀	2.3	16.0	11.5	9.0	9.0	9.5	14.5	14.5
5	♀	2.4	18.5	16.0	12.5	13.5	14.0	19.5	18.0
Average			18.7	15.2	12.8	13.0	13.0	16.5	17.9

TABLE VI

Blood Coagulation Time after Subcutaneous Injection of Elut of Spot B

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*win.*	*min.*
1	♂	2.3	19.0	17.5	13.0	14.0	13.5	12.0	13.5
2	♂	2.1	17.5	16.0	13.0	12.0	11.0	11.5	11.5
3	♀	2.1	18.5	17.5	14.5	13.0	11.5	13.0	12.5
Average			18.3	17.0	13.3	13.0	12.0	12.2	12.5

such solvents. Therefore, the author developed with the two phase solvent system of 5 per cent KH_2PO_4 and isoamylacohol (2:1), and now the Rf value of *Spot B* changed to 0.53. Such paper chromatographic behaviours coincide, according to Carter *et al.* (*4*), with that of adenosine or hypoxanthine. Therefore, a development was made simultaneously with adenosine and hypoxanthine as the controls, and all three showed the same Rf value. So, *Spot B* seemed to be adenosine or hypoxanthine or a mixture of these two. In order to distinguish them, various chemical tests and ultraviolet absorption spectra analysis were undertaken. In order to purify the *Spot B* elute for the purpose of these investigations (This is because, when developed only once, a little contamination with N_4 and N_5 is inevitable) the elute was concentrated and then developed again by the two phase solvent system; relevant paper section was cut and eluted with water.

Orcinol HCl Test: To each 0.5 ml of *Spot B* elute, 0.5 ml. of *Spot C* elute, and, as the control, 0.5 ml. of adenosine solution, was added 0.5 ml. of orcinol-HCl reagent (*5*), and heated 15 minutes in a boiling bath. No change was seen in *Spot B* elute and *Spot C* elute, but adenosine solution showed a nice green color. It means that nucleoside does not exist in *Spot B* elute.

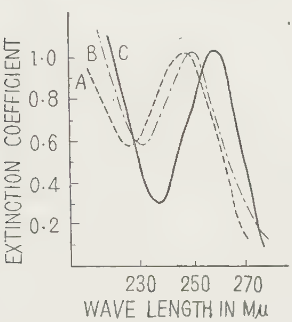
Hydrolysis: Each 0.25 ml. of 2 per cent H_2SO_4 solution was added to 0.5 ml. of *Spot B* elute and to the same amount of adenosine solution as a control, and put in a sealed tube and heated for one hour in a boiling bath, neutralized with barium carbonate, concentrated after filtration and developed on filter paper with *n*-butanol saturated with water. *Spot B* elute which was subjected to such a tratment yet showed the same Rf value of 0.26 as that of unhydrolysed *Spot B* elute and hypoxanthine. However, the hydrolysed solution of adenosine showed an Rf value of 0.4 which clearly changed to adenine. Thus, *Spot B*, through these investigations, could be identified as hypoxanthine.

Ultraviolet Absorption Spectra: From the above experiments, it was concluded that *Spot B* is hypoxanthine. However, in addition to this, an ultraviolet absorption spectra in acid, alkaline and neutral solutions were photographed and its absorption curve was studied. *Spot B* elute was diluted to 1.5 times and extinction from 0.0 to 1.5 was investigated with 10 mm.-thickness of the solution. As shown in Fig. 3, the $\lambda_{max.}$ is 248.5 mμ at pH 1.0, $\lambda_{max.}$ 250 mμ at pH 7.0, $\lambda_{max.}$ 261.5 mμ at pH 10.0 which are identical with that of hypoxanthine (*6*).

Spot A—Owing to a small amount of substances contained in the A fraction, together with the increase of the Rf value based upon using

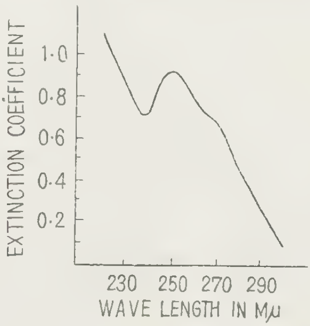
the two phase solvent system, the detection was very difficult due to the diffusion of the spot. However, by several developments and with the aid of spraying the fluorecein, it was confirmed that this spot consisted of two substances having Rf values of 0.60 (*Spot* A_1) and 0.69 (*Spot* A_2). As these seemed to correspond to Rf values of guanosine and inosine, respectively, a smiultaneous development of the test substances was performed with pure guanosine and inosine, and their identity was confirmed. Therefore, the following experiments were carried out with the elutes of *Spots* A_1 and A_2 for the further confirmation.

FIG. 3. Ulta violet absorption curve of *Spot B*



A : $\lambda_{max.}$ 248.5 mμ at pH 1.0
B : $\lambda_{max.}$ 250.0 mμ at pH 7.0
C : $\lambda_{max.}$ 261.5 mμ at pH 10.0

FIG. 4. Ulta violel absorption curve of *Spot A*



$\lambda_{max.}$ 250 mμ at pH 7.0

Orcinol HCl Test: The method used is the same as in the case of *Spot B*. The results were as follows:

Elute of *Spot* A_1	Positive (+)
Elute of *Spot* A_2	Positive (+)
Elute of *Spot B*	Negative (−)
Guanosine solution	Positive (+)

These results indicates that nucleoside is contained in both *Spot* A_1 and *Spot* A_2 elutes.

Hydrolysis: When the hydrolysed solution of *Spot A* elute was developed with *n*-butanol saturated with water as solvent, two spots with Rf values of 0.05 and 0.26 were seen which coincide with the Rf values of hydrolysed solution of guanosine and inosine mixture and

consequently with the Rf values of guanine and hypoxanthine, respectively.

Ultraviolet Absorption Spectra: The ultraviolet absorption spectral curve of *Spot A* at pH 7.0 coincides very well with that of the mixture solution of guanosine and inosine (*6*, *7*) in ratio of 3:1. As shown in Fig. 4 the $\lambda_{max.}$ is 250 mμ.

Spot C—C fraction which does not accelerate blood coagulation, indicated that it is no more than uracil because it gave no orcinol-HCl test and showed the same Rf values as that of uracil in *n*-butanol saturated with water and in the two phase solvent of 5 per cent KH_2PO_4 and iso-amylalcohol, and the identical ultraviolet absorption spectra in acid and alkaline as that of uracil. The Rf value showed 0.38 in butanol system, and 0.68 in the two phase solvent system.

SUMMARY

The author was able to separate hypoxanthine, inosine and guanosine as the active principles from the bone marrow extract which showed a remarkable accelerating action on the blood coagulation by paper chromatography. This was done by cutting the whole filter paper in several sections separately along the Rf values of the detected spots, eluting them with water, and investigating their physiological action. Then the chemical nature of the substances which were contained in the elutes from the A and B fractions having accelerating action on the blood coagulation was investigated with various tests.

The author would like to extend his appreciation to Prof. Katashi Makino for his kind guidance and also to Dr. Kiyoo Satoh for his cooperation in reading the ultraviolet absorption spectra.

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ON THE CHEMICAL NATURE OF BLOOD COAGULATION ACCELERATORS CONTAINED IN THE SPLEEN EXTRACT

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(Received for publication, June 22, 1953)

In the previous papaer (*1*) it has been reported that blood coagulation accelerators contained in the bone marrow extract are hypoxanthine, inosine and guanosine. In this report, investigations on the spleen extract will be described. It is well known that in 1904 Landau (*2*), for the first time, used spleen extract for hemostatic purpose and recognized that it has an action to accelerate blood coagulation. From the fact, that the same effect can be obtained by a short-time irradiation of X-ray on the spleen of a patient having hemorrhagic diathesis, it was considered by Neuffer *et al.* (*3*) that the function of the reticulo-endothelial system in the spleen was stimulated and as a consequence of this the blood coagulation was accelerated. According to Hoffmann (*4*), it is due to the production of a new constituent by destruction of the leucocyte and lymphocyte in the spleen by the irradiation of X-ray. But the chemical nature of its principle is also still obscure. Therefore, the author conducted an experiment using spleen extract (Opostatin) in the same manner as in the case of bone marrow extract. From these results, it was found that hypoxanthine and inosine are also contained as the blood coagulation accelerators in the spleen extract. As these two substances besides guanosine were also found in the bone marrow extract as described above, the author conducted animal experiment with the pure samples of hypoxanthine, inosine and guanosine and could confirm that these substances have remarkable blood coagulation accelerating action, but they were effective only *in vivo* and no activity could be detected *in vitro*.

EXPERIMENTAL

Material

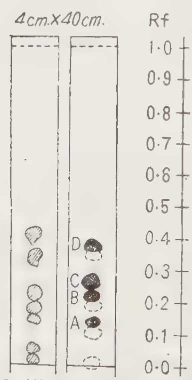
A spleen extract preparation called " Opostatin " was used, which

was made from fresh spleen of animal, and 1 ml. of this preparation is said to correspond to 5 g. of fresh spleen. As shown in Table I, the activity of Opostatin is a little weaker than that of Medullan in the same doses. 20. ml. of this preparation were concentrated to a small volume *in vacuo* at low temperature, then dried to dryness in a vacuum desiccator over calcium chloride and treated with alcohol three times for desaltation. Finally, the alcoholic solution was concentrated to approximately 0.2 ml.

Separation of Substances by Paper Chromatography

The syrup-like material concentrated and desalted as described in the foregoing section, was developed on a No. 50 Toyo filter papers measuring 4 cm. ×40 cm. and 40 cm. ×40 cm. with *n*-butanol saturated with water, the former being used to detect the Rf values of spots and the latter for the purpose to make fractional elution. In this case, as shown in Fig. 1, four spots of A, B, C and D with Rf values of 0.14, 0.22, 0.26 and 0.38, respectively, and other four spots with Rf values of 0.01, 0.1, 0.18 and 0.35 showing a light purple fluorescence were found with the ultraviolet light detector. Moreover, seven spots with Rf values of 0.01, 0.05, 0.16, 0.19, 0.22, 0.34 and 0.40 were detected with ninhydrin, indicating the existence of several amino acids. The wider filter paper which was developed as described above was cut off corresponing to the individual Rf, as shown in Fig. 2, and then elutes were made from every section and concentrated to 2 ml. each, and used for

FIG. 1



: Ninhydrin positive Spots
: Fluorescent Spots

FIG. 2

*. No. of section to be cut off for elution

animal experiment.

Effects of Separated Fractions upon Blood Coagulation

In order to study the effects upon blood coagulation of the fractions separated from the spleen extract, animal experiment was performed in the same manner as in the case of bone marrow extract. Blood coagulation time was measured after injecting 1 ml. of each elute to rabbits subcutaneously.

The results are shown in Tables I∼IV ,and the elutes from the A and C fractions were found to have distinct accelerating action on the blood coagulation, whereas the remaining seven fractions showed no activity.

TABLE I

Blood Coagulation Time after Subcutaneous Injection of Opostatin

Rabbit No.	Sex	Body weigyt	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*miu.*	*min.*
1	♀	2.5	18.5	19.5	18.0	15.5	14.0	13.5	15.5
2	♂	2.3	18.0	16.5	14.0	12.0	12.5	12.5	13.5
Average			18.3	18.0	16.0	13.8	13.3	13.0	14.5

TABLE II

Blood Coagulation Time after Subcutaneous Injection of Elute of Spot D

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♀	2.1	19.5	15.5	19.5	20.0	21.0	19.0	19.5
2	♂	2.4	18.0	19.5	18.5	17.0	19.0	20.0	18.5
3	♂	2.3	15.0	16.0	15.5	14.5	15.5	14.5	15.5
Average			17.5	17.0	17.8	17.2	18.5	17.8	17.8

Chemical Nature of Spots A B C, and D—In order to identify the substances contained in the A and C fractions accelerating the blood coagulation and also in the B and D fractions without activity, the author performed investigations in the same manner as in the case of bone

TABLE III

Blood Coagulation Time after Subcutaneous Injection of Elute of Spot A

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	190
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min*
1	♂	2.5	18.5	17.5	15.0	12.5	16.0	12.0	12.5
2	♀	2.4	16.5	17.0	13.0	11.0	12.0	12.0	12.5
3	♂	2.5	18.0	16.5	12.0	12.5	12.0	12.5	13.5
Average			17.7	17.0	13.3	12.0	13.3	12.2	13.8

TABLE IV

Blood Coagulation Time after Subcutaneous Injection of Elute of Spot C

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♂	2.1	17.5	17.0	13.5	11.5	12.5	12.0	14.5
2	♀	2.5	20.0	17.0	12.5	14.0	12.0	14.0	13.5
3	♂	2.5	18.5	15.5	13.5	12.0	12.0	13.5	15.0
Average			18.7	16.5	13.2	12.5	12.2	13.2	14.3

TABLE V

		Spot A	*Spot C*	Inosine	Hypoxanthine
Rf-values with *n*-butanol water system		0.14	0.26	0.14	0.26
Rf-values with two phase solvent system of Carter (*5*)		0.68	0.53	0.68	0.53
Rf-values after acidic hydrolysis (with *n*-butanol water system)		0.26		0.26	0.26
Orcinol-HCl test (*6*)		+	−	+	−
Absorption maxima in mμ (*7*)	pH 1.0	249	248	248	249
	pH 7.0	250	249	250	250
	pH 10.0	254	261	254	262

marrow extract (*1*). The results are briefly summaized in Table V.

Thus it becomes clear that the blood coagulation accelerators contained in the spleen extract are also hypoxanthine and inosine. *Spots B* and *D* are found to be uridine and uracil, respectively.

Effects of Pure Hypoxanthine, Inosine and Guanosine upon Blood Coagulation

Since it has become clear that blood coagulation accelerators contained in the bone marrow and spleen extracts are hypoxanthine, inosine and guanosine, the author conducted animal experiment *in vivo* and *in vitro* using these samples.

The sample of hypoxanthine was an authentic product of the Schwarz Laboratory, and inosine and guanosine were prepared in our laboratory.

Experiments in vivo—In physiological tests the author used the Sahli-Fonio method modified by Tanaka (*8*) at 20°, with rabbits weighing 2.0～2.5 kg. as test animals. Each 200 γ of hypoxanthine, inosine and guanosine dissolved in 2 ml. of water separately, and 2ml. of water as a control, were injected subcutaneously to each animal and the time required for blood coagulation after the injection was measured every half hour, for 3 hours.

As seen from the results in Tables VI～IX, the action of hypoxanthine began at 30 minutes after the injection and the action of inosine and guanosine began at 60 minutes after the injection. The most remarkable action was seen at 90～120 minutes after the injection but even after 180 minutes, the action still remained. The activity of inosine seems a little weaker than that of hypoxanthine or guanosine.

Experiments in vitro—In order to investigate the action *in vitro* of the test substances the author made experiments as follows. 4 ml. of blood were withdrawn from the ear vein of a healthy rabbit with a syringe containing 0.4 ml. of 1.2 per cent potassium oxalate as anticoagulant. After centrifugation, each 0.2 ml. of oxalate blood plasma was taken into a pipette and transferred unto two watch glasses. Then one of them was mixed with 0.2 ml. of 0.025 *M* calcium chloride solution containing each 20 γ of test substances while the other as the control was mixed with 0.2 ml. of 0.025 *M* calcium chloride solution without test substances. Each of them was mixed well with a glass rod and the time required for the production of the fibrin was checked, and then the time for the disappearance of liquidity was measured. How-

ever, no coagulation accelerating action of purine derivatives was noticed, the time required for blood coagulation at 20° having been 10~11 minutes, whether these substances were added or not.

Therefore, hypoxanthine, inosine and guanosine are effective in the acceleration of blood coagulation only when they are injected.

TABLE VI

Blood Coagulation Time after Subcutaneous Injection of Hypoxanthine

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*uin.*	*min.*
1	♂	2.4	18.0	13.0	12.5	12.0	11.5	12.0	14.5
2	♂	2.5	18.0	18.0	12.5	11.5	11.0	12.5	15.5
3	♀	2.3	18.0	15.5	15.0	11.5	13.0	15.5	14.0
Average			18.2	15.5	13.3	11.7	11.8	13.3	14.7

TABLE VII

Blood Coagulation Time after Subcutaneous Injection of Inosine

Rabcit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♀	2.1	14.0	13.5	9.5	10.0	9.5	9.0	10.0
2	♂	2.3	18.5	16.0	14.0	12.5	16.0	14.5	14.5
3	♀	2.5	17.0	16.5	16.0	14.0	12.5	16.0	13.5
Average			16.5	15.3	13.2	12.2	12.7	13.2	12.7

TABLE VIII

Blood Coagulation Time after Subcutaneous Injection Guanosine

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♀	2.3	16.5	16.0	12.5	10.0	12.5	11.5	12.5
2	♂	2.5	17.5	16.0	12.5	12.0	14.0	13.5	14.5
3	♀	2.5	19.0	17.5	13.5	12.5	11.0	14.0	14.5
Average			17.7	16.5	12.8	11.5	12.5	13.0	13.8

TABLE IX

Blood Coagulation Time after Subcutaneous Injection of Distilled Water

Rabbit No.	Sex	Body weight	Before injection	Time after injection in minutes					
				30	60	90	120	150	180
		kg.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
1	♂	2.3	18.5	19.0	17.0	19.0	20.0	18.0	17.5
2	♀	2.5	17.0	17.0	19.5	18.0	17.5	18.0	18.5
3	♀	2.5	20.0	18.5	18.0	18.5	19.5	17.5	17.0
Average			18.5	18.2	18.2	18.5	19.0	17.8	17.7

SUMMARY

The author was able to separate hypoxanthine and inosine as the blood coagulation accelerators from the spleen extract by paper chromatography and identified by the paperchromatographic and ultraviolet spectrophotometric technics. Moreover, the author described the remarkable accelerating action on the blood coagulation of pure hypoxanthine, inosine and guanosine and recognized that they are effective only *in vivo* but not *in vitro*.

The author would like to extend his appreciation to Prof. Katashi Makino for his kind guidance and also to Dr. Kiyoo Satoh for his cooperation in reading the ultraviolet absorption spectra.

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The Journal of Biochemistry, Vol. 40, No. 5, 1953

STUDIES ON THE ENZYMATIC BREAKDOWN OF LIMITDEXTRIN.

I. THE ENZYMATIC BREAKDOWN OF β-LIMITDEXTRIN BY α-AMYLASE

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(Received for publication, July 2, 1953)

It was first reported by Meyer (*1*) that starch consists of two fractions, amylose of a linear unbranched chain structure and amylopectin of a branched chain stucture. A good deal of work has been done on the action of amylase on these two compounds. Myrbäck (*2*) has pointed out that in general, starch and glycogen are not completely degraded to fermentable sugars by amylase; the saccharification ceases at a more or less definite stage, leaving non-fermentable substances called "limitdextrin," "stable dextrin" or "residual dextrin".

The incomplete saccharification of starch is due neither to the reversiblity of the reaction, nor to the dynamic equilibrium, but rather to the existence of some abnormal constitution in the chain molecules differing from that of the bulk of the polysaccharide.

To get more clear undersanding on the nature of such abnormal constitution which is refractory to the action of amylase, the author studied the action of α-amylase on β-limitdextrin, which was obtained as the final products of β-amylase action on amylopectin, using potentiometric titration method by iodine. (*3*, *4*, *5*).

EXPERIMENTAL

The Preparation of Enzymes—As the materials for obtaining α-amylase and β-amylase, human saliva and ungerminated barley were used respectively. α-Amylase was prepared by Meyer's method (*6*), β-amylase by the method of Caldwell (*7*) and the purity of such enzyme preparations was tested by following changes of reducing power of products and also by potentiometric titration curves during its action on amylose.

The Separation of Amylose and Amylopectin Fractions of Starch and the Preparation of β-Limitdextrin—Amylose was prepared from potato starch according to Schoch's butanol precipitation method (*8*) and further purified by two succesive crystallizations from the boiling butanol-water mixture. Amylopectin was prepared from Schoch's amylopectin

fraction by the method of Tanrct and Pascu (*9*). The purity of the prepared amylose was tested by the potentiometric titration with the result quite the same as that of Rundle (*3*) obtained on Kerr's crystalline amylose. The prepared amylopectin was proved to be free from contamination of amylose by the reddish purple color of the iodine reaction and further by the shape of the potentiometric titration curve.

β-Limitdextrin was prepared by the following method (*11*): A complete dispersion of 500 mg. of purified amylopectin in 5 ml. of 5 *N* KOH was diluted with water, then neutralized by the addition of dilute HCl with methyl red as an indicator, and finally diluted to 100 ml. with water. 10 ml. of freshly prepared β-amylase solution were added to the mixture of 100 ml. of the amylopectin solution, 30 ml. of *M*/10 acetate buffer (pH 4.6,) 10 ml. of 2 per cent NaCl solution and 150 ml. redistilled water. The reaction mixture was incubated in a thermostat at 37° under toluene. The iodine reaction of the incubated solution changed from purple to red purple. After 8 or 10 hours its reducing power reached a constant value (approximately 55.7 per cent of the original amylopectin as maltose). The reaction was stopped by heating in a boiling water bath, the mixture was filtered and precipitated by adding an equal volume of absolute alcohol. The precipitate was centrifuged, washed several times with 50 per cent alcohol, and dried on anhydrous calcium chloride.

The precipitate was the limit for the action of the β-amylase under the same experimental conditions. It gave an iodine-reaction of red purple.

According to Ogata (3) the potentiometric measurement of the iodine-reaction indicates that 16 mg. of amylopectin are equivalent to 8 mg. of β-limitdextrin. Therefore, curve B in Fig. 1 indicates that 60 mg. of amylopectin was changed to 16 mg. of amylopectin, equivalent to 8 mg. of β-limitdextrin by the action of β-amylase.

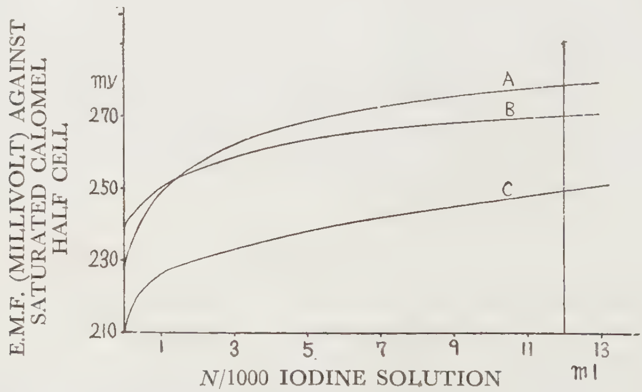

FIG. 1. The action of β-amylase on amylopectin (60 mg./dl.); the iodine titration curves at final reaction time.

A. $KI-I_2$ titration curve (blank).

B. The titration curve of 60 mg. of amylopectin which was digested by the action of β-amylase.

C. The titration curve of amylopectin (60 mg./dl.) (original amylopectin).

The Determination of Amylopectin and β-Limitdextrin—The determination was carried out according to Ogata (2). The final volume of the titrated solution was 50 ml. and titration was carried out under vigorous aeration with $N/1000$ iodine solution containing the same concentration of potassium iodide. For the titration 0.2 ml. of $N/10000$ iodine solution were dropped at 2 minute intervals. After each addition the potential of a platinum electrode inserted into the titrated solution was measured against a saturated calomel electrode using a potentiometer of the Cambridge unipivot type.

The Measurement of Reducing Power—The reducing power of the reaction product was estimated by the method of Hagedorn and Jensen (*10*).

The Experimental Conditions—The action of α-amylase on β-limitdextrin was investigated under the following conditions. One ml. of freshly prepared enzyme solution was added to the mixture of 3 ml. of the substrate solution, 2 ml. of 2 per cent NaCl solution, 2 ml. of 1/10 M phosphate buffer at pH 6.4, and 2 ml. of redistilled water. The substrate solution was prepared as follows. A weighed amount of substrate was completely dispersed in 10 ml. of 5 N KOH solution diluted with water, neutralized by the addition of HCl, with methyl red as indicator, and was finally diluted to a definite volume. For the potentiometric titration this reaction mixture was treated as follows: 5 ml. of 5 N KOH solution were added to each reaction mixture at regular intervals to stop the enzyme action, the mixture was transferred quantitatively to a Michaelis's vessel, diluted with water, adjusted to pH 3 with 0.5 N HCl just before the beginnin of the titration, filled up with water to 45 ml. and finally 5 ml. of 0.5 N potassium iodide

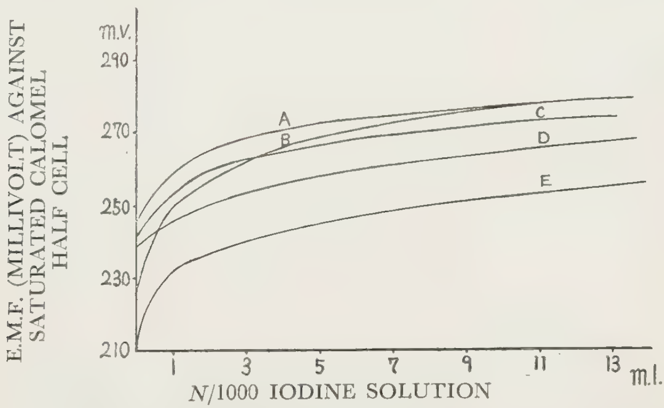

FIG. 2. The action of α-amylase on β-limitdextrin; the iodine titration curves at various reaction times.

These curves indicate the change of titration curves taking place with the progress of enzyme reaction.

A: Reaction time, 35 minutes. B: KI-I_2 titration curve (blank). C: Reaction times, 15 minutes. D: Reaction time, 5 minutes. E: Original β-limitdextrin (30 mg).

solution were added for the potentiometric titration. For the measurement of reducing power of the reaction mixture, 0.1 ml. was pipetted out at the same regular intervals.

RESULTS AND DISCUSSION

The Action of α-Amylase on β-Limitdextrin—As described by K. H. Meyer, the all end chains in the β-limitdextrin are derived from amylopectin by the action of β-amylase. Accordingly the outermost linkages of β-limitdextrin are anomalous linkages (α-1,6-linkage), otherwise the second linkages from out side are anomalous. Therefore, if α-amylase can split β-limitdextrin the action of α-amylase which does not act on the anomalous linkages (α-1,6-linkage) must attack only the internal normal chains (α-1,4-linkage). The potentiometric titration curves

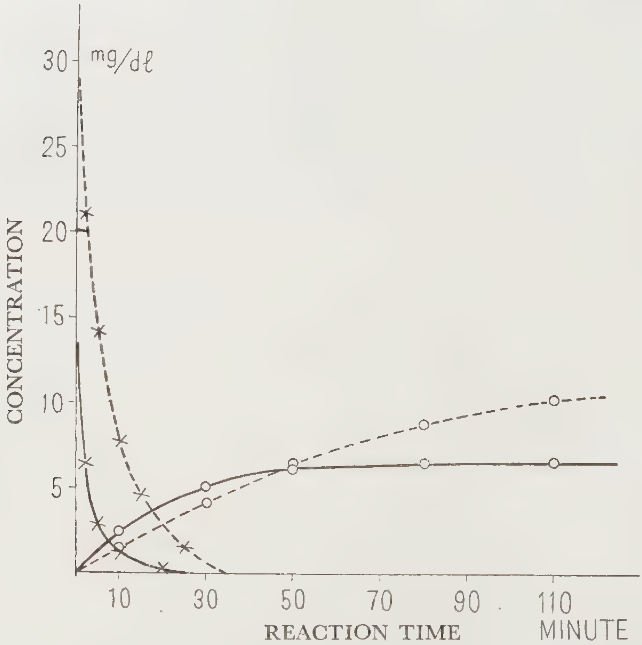

FIG. 3. The action of α-amylase on β-limitdextrin; the relationship between the remaining β-limitdextrin and the produced glucose.

× β-Limitdextrin, ○ Maltose. The solid lines indicate the case when the substrate concentration was 15 mg./dl. The broken lines indicate the case when the substrate concentration was 30 mg./dl.

(Fig. 2) indicated that the type of these curves was similar to that obtained by the author with amylopectin. With the advancement of the enzyme reaction, the iodine reaction changed gradually from red-purple (α-limitdextrin) to orange and further to yellow. It may be inferred from the results that the relationship between the action of α-amylase and the structure of β-limitdextrin is the same as that between the action of α-amylase and the structure of amylopectin. Namely, α-amylase, as Myrbäck reported already, splits the internal normal linkages of the polysaccharide molecules independently of anomalous linkages in all end chains. The amount of β-limitedextrin was estimated by the potentiometric titration and the maltose produced in the reaction mixture was estimated by the reducing power of the mixture at various points of time. As shown in Fig. 3 the symmetrical curves were obtained for both concentrations of β-limitdextrin (15 mg./dl. and 30 mg./dl.).

SUMMARY

β-Limitdextrin was prepared from amylopectin by the action of barely β-amylase. The action of salivary α-amylase on β-limitdextrin was examined by the iodine potentiometric titration method. The titration curves showed to be of the same type as that of amylopectin. It was demonstrated that the salivary α-amylase split the internal α-1,4-linkages in amylopectin and β-limitdextrin in the same way independently of the α-1,6-linkage of all end chaine.

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The Journal of Biochemistry, Vol. 40, No. 5, 1953

STUDIES ON THE ENZYMATIC BREAKDOWN OF LIMITDEXTRIN.

II. THE α-LIMITDEXTRIN

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(Received for publication, July 2, 1953)

As described in the previous report (*1*), amylopectin and β-limitdextrin are not completely transformed into fermentable sugars by α-amylase, but a definite limit of saccharification is reached, giving α-limitdextrin as residues (*2*, *3*, *4*). In the present paper, some properties of α-limitdextrin are reported, which was prepared from amylopectin by the action of salivary α-amylase.

EXPERIMENTAL

The Preparation of α-Limitdextrin—One g. of amylopectin was completely dispersed in 5 ml. of 5 N KOH solution and the dispersed solution was diluted with water, neutralized by HCl with methyl red as an indicator, and finally diluted to 200 ml., 20 ml. of freshly prepared α-amylase solution was added to the mixture of 200 ml. of diluted amylopectin solution, 40 ml. of 1/10 M phosphate buffer of pH 6.4, 20 ml. of 2 per cent NaCl solution and 20 ml. of redistilled water. Then several drops of toluene were added and the solution was incubated in a thermostat at 37°.

After the reducing power had reached (84 per cent of the original amylopectin as maltose) a constant value, the incubated solution was heated in a boiling water bath for 20 minutes, cooled, and filtered. The filtrate was condensed in vacuo to 20 ml. to which was added absolute alcohol to a concentration of 95 per cent. After standing over night the precipitates formed were collected and dried on anhydrous calcium chloride.

The Porperties of α-Limitdextrin—

(a) The α-limitdextrin was found to be refractory to a prolonged action of α-amylase for 48 hours under the same conditions.

(b) The rate of acid hydrolysis: The conditions of the hydrolysis were almost the same as those of Wolfrom and Oeneel (*5*), except that hydrochloric acid was used instead of sulfuric acid. The concentration of the amylopectin was 0.2 per cent. The final concentration of hydrochloric acid was 0.05 N. The temperature was mainained at 99.5° in a boiling water-bath. Samples were taken at suitable intervals, neut-

ralized with NaOH, and diluted to a definite volume.

The degree of hydrolysis was determined from the reducing power by Hagedorn Jensen's method (*6*). The rate constant was calculated according to the following equation: $K=\frac{1}{t}\log_{10}\frac{c}{c-x}$, where c=glucose found on complete hydrolysis, x=the reducing power in terms of glucose expressed in per cent of c, and t=time in minutes.

TABLE I

Hydrolysis Constant on 0.05 *N HCl at* 99.5°
(The concentration of polysaccharide was 0.2 per cent)

Time	α-Limitdextrin		β-Limitdextrin	
	Hydrolysis	$K\times10^3$	Hydrolysis	$K\times10^3$
min.	*per cent*		*per cent*	
120	15	0.592	30	1.29
240	25.8	0.545	45	1.08
360	34.5	0.520	56	0.99
480	43.5	0.517	66.5	0.99
	Average	0.543	Average	1.12

TABLE II

Hydrolysis Constants of Various Polysaccharides in 0.05 *N HCl at* 99.5°
(The concentration of polysacchrides was 0.2 per cent)

Polysaccharides	$K\times10^3$
Amylose	1.76
Amylopectin	1.54
β-Limitdextrin	1.12
α-Limitdextrin	0.54

The rate constants obtained with amylose, amylopectin, β-limitdextrin and α-limitdextrin are summarized in Tables I and II, in which the relation of the hydrolysis constant to the structure of these polysaccharides may be recognized; as may be seen from these tables, amylose (linear polymers containing only α-1,4-linkages) shows the highest rate 1.76 and amylopectin (branched polysaccharides containing both α-1,4- and α-1,6-linkages) 1.54 and β-limitdextrin (branched polysaccharides smaller than the former containing both α-1,4- and α-1,6-linkages) 1.12, and α-limitdextrin (almost all linkages are α-1,6 linkages) the lowest rate, 0.54.

As reported by Cori and Swanson (*7*), and Wolfrom (*5*), the α-1,4 linkage is definitely more easily hydrolyzed than the α-1,6-linkage. The ratio of the acid hydrolysis constant for maltose to isomaltose was 0.195: 0.048. The rate constants for amylose and α-limitdextrin obtained by the author were 1.76 and 0.543, rescectively.

Thus, α-limitdextrin is supposed to be the mixture of isomaltose, triose and a larger sacharide molecule which contains the α-1,6-glucosidic linkages.

c) Paperchromatography of α-limitdextrin: The test was carried out by modified method of Cori (*8*). Chromatography showed the presence of two or three reducing sugars, which were detected by spraying alkaline copper sulfate solution upon the paper and heating for 15 minutes at 100°. These sugars were proved to be triose, isomaltose, or other large reducing sugars by their Rf values. The Rf values under such experimental condition were 0.23 for the 1st sugar of the limitdextrin and 0.15 for the second, while glucose and maltose showed the values 0.37 and 0.26, respectively.

SUMMARY

α-Limitdextrin was prepared from amylopectin by the action of α-amylase. Its properties were studied.

1. α-Limitdextrin was stable against α-amylase for 48 hours.
2. The ratio of the rate constant of acid hydrolysis of α-limitdextrin to that of amylose was about 1:3.
3. It was recognized by paper chromatography that α-limitdextrin consisted of two or three kinds of reducing sugars.

These results indicate that the α-limitdextrin obtained by the author contains the compounds consisting mainly of α-1,6-linkages.

The author wishes to express his gratitude to Prof. K. Kodama and Prof. H. Yoshikawa for their reading of the manuscript and kind advices. His thanks are also due to Mr. M. Hosoya for his valuable technical assistance in this study.

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STUDIES ON DIGESTION OF STARCH BY α-LIMITDEXTRINASE

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(Received for publication, July 2, 1953)

It is usually assumed that starch is broken down into maltose by the action of ptyalin in saliva and amylopsin in pancreatic juice and the resulted maltose into glucose by matltase secreted from intestinal mucosa. It is also postulated that both ptyalin and amylopsin are chiefly of the nature of α-amylase type and maltase is of α-glucosidase type which is able to split α-1,4-linkage of α-maltose. But it was shown in the foregoing report (*1*) that amylopectin can not be splitted completely into maltose by ptyalin leaving some fraction of dextrins which is quite resistent to α-amylase. We call this substance as α-limitdextrin (*1*, *2*, *3*). To judge from its attitude toward acid hydrolysis, this α-limitdextrin comprises much of α-1,6 glucosidic linkage (*2*). To achieve the complete hydrolysis of this linkage there should be some enzyme of another type in intestinal juice. The existence of such an enzyme in plant has been proved and it is designated as isomaltase. (*4*, *5*, *6*, *7*, *8*, *9*). But the existence of isomaltase in animal tissue remains still obscure at present. The author has, therefore, conducted a search for this sort of enzyme and it was found that in intestinal juice and also in intestinal mucosa an enzyme exists which can split α-limitdextrin into glucose. Since pancreatic tissue contains no such enzyme it seems rational to conclude that the enzyme is secreted from intestinal mucosa. Many trials to purify this enzyme were executed, but the preparations so far obtained always accompanied maltase, so that it was unable to decide whether this enzyme is a specific one or not. It remains also undecided whether this enzyme is identical with isomaltase or not. For a moment we call this enzyme tentatively α-limitdextrinase, until a more precise information can be obtained. At any rate the complete digestion of starch into glucose can be accomplished by the aid of this enzyme in cooperation with ptyalin, amylopsin and maltase, so that the importance of this enzyme cannot be overemphasized.

EXPERIMENTALS

Preparation of the Enzyme from the Bovine Intestinal Juice (Preparation A)—The fresh juice collected at the slaughter house was diluted twice with water and centrifuged. The supernatant was filtered through paper pulp in Buchner's funnel. To the clear yellowish filtrate acetone was added up to 40 per cent. After removing the precipitate acetone was added further to 60 per cent. The second precipitate was dissolved in a small amount of water and again precipitated by the addition of ammonium sulfate up to 45 per cent saturation. The precipitate was washed with ammonium sulfate of the same concentration, dried in a desiccator and preserved therein.

Preparation of the Enzyme from the Intestinal Mucosa. (Preparation B)—The fresh bovine intestinal mucosa was treated with twice its volumes of acetone, 3 times with ether-acetone (1:1) mixture and 3 times with ether, succesively, to bring about complete-defatting and drying, and preserved in a desiccator over silica gel *in vacuo*. Upon me, this powdered preparation was extracted with 1/20 *M* sodium acetate solutions under occasional shakings. The extract was once passed through paper pulp in Buchner's funnel and the clear filtrate was treated with acetone and precipitated by ammonium sulfate in the same manner as described in the preparation from juice.

Activity of the Enzyme at Various Stages of Purification—The enzyme activity was tested by mixing 2 ml. of the enzyme solution, 1 ml. of 2 per cent NaCl solution, 3 ml. of 0.1 per cent substrate solution, 2 ml. of phosphate buffer at pH 7.0 and 2 ml. of distilled water. The whole mixture was incubated at 37° for 24 hours and the increase of reducing power (represented as glucose) was determined by the method of Hagedorn and Jensen (*10*). As the substrates the purified amylose (*1, 11, 12*), maltose (*1*) and α-limitdextrin (*1, 13*) were used.

The results are given in Table I, in which increased value of glucose is indicated as percentage of that of complete hydrolysis.

From Table I we can see that by treating with acetone and ammonium sulfate amylase activity was more reduced than α-limitdextrinase and maltose, though it was unable to get rid of it completely.

TABLE I

Enzyme Activity of Preparation B at Various Stages of Purification

Stage of purification	Protein contents in 100 ml. of enzyme solution.	Hydrolysis of substrates (Percent as glucose)		
		α-Limit-dextrin	Amylose	Maltose
	mg.	*per cent*	*per cent*	*per cent*
Buchner's funnel filtrate (Prepn. I)	620	21.4	18.0	40.0
Acetone precipitate (Prepn. II)	42.5	12.4	15.0	17.8
Ammonium sulfate precipitate (Prepn. III)	38.5	12.4	8.3	17.0

The enzyme activity was tested at 37° for 24 hours and pH value was 7.0.

The question whether α-limitdextrinase and maltase are different enzymes or not is difficult to decide, but there seems to exist a certain tendency that both enzymes are different, as maltase activity was somewhat more lessened than α-limitdextrinase by those treatments. Namely maltase activity was reduced to 42.5 per cent and α-limitdextrinase to 57.9 per cent.

The Effect of Heat on the Stability of the Enzymes—The purified *Preparation B* was dissolved in test tubes and kept in water baths at 50°, 60° and 70° for 10 minutes and then its maltase, α-limitdextrinase and amylase activities were tested as usual. The results are shown in Table II.

TABLE II

The Effect of Heat on the Enzymatic Activities for Various Substrates

(The enzyme activity is represented by hydrolysis percentage calculated as glucose.)

Substrate \ Temperature	Room temperature	50°	60°	70°
	per cent	*per cent*	*per cent*	*per cent*
Maltose	87.9	85.7	54.5	28.9
α-Limit-dextrin	50.5	48.3	25.8	11.2
Amylose	27.4	18.7	4.3	3.3

The result obtained indicates that amylase is more labil than maltase and α-limitdextrinase, since the activity of amylase retained only 3.3 per cent after being exposed to 70°, while maltase and α-limitdextrinase 29 per cent and 11 per cent, respectively. It should be noticed, however, that the heat stabilities of maltase and α-limitdextrinase are not the same.

The Effect of Salts upon the Actions of Enzymes—Both enzyme solutions obtained by dissolving *Preparatious A* and *B* in water were dialyzed against redistilled water at

TABLE III

The Effect of NaCl upon the Actions of Dialyzed Enzyme Preparations

(The action of enzyme is indicated as hydrolysis percentage of substrate as glucose.)

Enzyme preparation	NaCl	Substrate hydrolyzed		
		Amylose	α-Limitdextrin	Maltose
		per cent	*per cent*	*per cent*
A, dialyzed	None	57.8	51.2	90
,, ,,	Added	79.0	58.5	90
B, dialyzed	None	33.6	53.9	84
,, ,,	Added	36.0	51.7	84

0° for 60 hours. Into one portion of the dialyzed solutions NaCl was added to a concentration of 0.2 per cent, and enzyme activities were compared with those without the addition of NaCl. The results are given in Table III.

We can see from the above data that α-limitdextrinase and maltase are not so much influenced as amylase by the presence of salt.

The Effect of Acidity on the Actions of Enzymes—The enzyme solutions were acidified with tartarate buffer to pH 2.2 and 3.0, and left for 30 or 60 hours at 5°, neutralized again, and their activities were measured at optimal pH's values, respectively (see the following experiment). The results are given in Table IV.

It is apparent from the table that the enzymes in *Preparation A* are more sensible to acid than those in *Preparation B*. It is also noticeable that amylase in *Preparation B*

TABLE IV

The Effect of Acidity

(The action of enzyme is indicated as hydrolysis percentage of substrate as glucose)

Substrate hydrolyzed	pH 2.2				pH 3.0			
	30 hrs.		60 hrs.		30 hrs.		60 hrs.	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Maltose	0	71.0	0	—	41.1	87.9	43.9	—
α-Limitdextrin	1	51.8	0	—	34.1	50.5	34.1	—
Amylose	4.6	5	0	—	44.7	27.4	34.7	—

A: *Preparation A.* B: *Preparation B.*

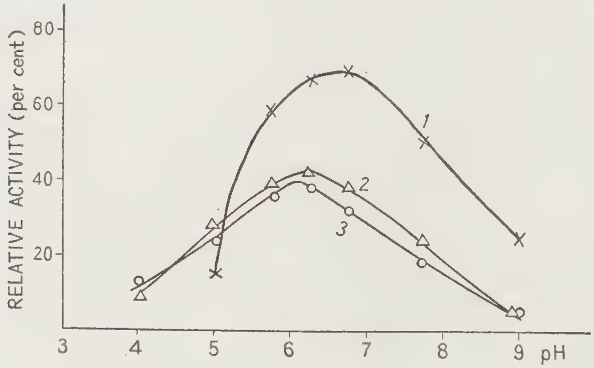

FIG. 1. The relative activity of the *Preparation A* at different pH values.
1. Amylose 2. α-Limitdextrin 3. Maltose

is more labile to high acidity.

Optimal pH—The enzyme solution was brought to various pH by adding veronal buffer. The results are indicated in Fig. 1. Here we can see that the optimal pH of amylase was 6.7 and those of maltase and α-limitdextrinase near 6.

The Electrophoretical Pattern of Preparation B—The author observed the electrophoretic distribution of enzyme proteins obtained by the method of Young (*14*). The enzyme powder was dissolved in a small amount of buffer and dialyzed in a cellophane tube for 24 hours at 0° against 500 ml. of buffer solution. The buffer solution used was *M*/20 phosphate buffer at pH 8.1 and ionic strength 0.14. The electric current was stabilized at 9.3 mA. and the time of electrophoresis was 15 minutes. The results given in Fig. 2. shows that the preparation consists of four components.

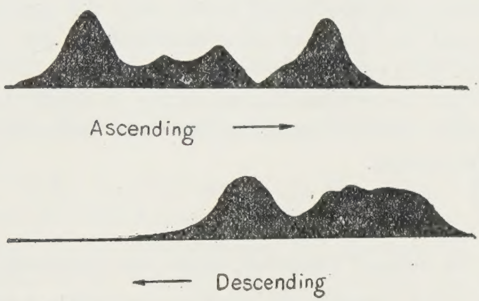

FIG. 2. Electrophoretic diagrams of *Preparation B.*

DISCUSSION

Though the hydrolysis was not complete because of the crudeness of the enzyme preparation, α-limitdextrin, maltose and amylose were surely broken down; therefore the starch can be digested almost completely in intestinum. The existence of α-amylase may probably be due to the contamination of pancreatic α-amylase, for the iodine-reaction of amylose changed gradually from blue to purpul and finally to red and its properties (*2*, *3*, *16*, *15*) were similar to pancreatic α-amylase.

To remove α-amylase from the enzyme preparation, the starch-absorption technique was applied as follows (*17*, *18*, *19*, *20*); to 200 ml. of cold *Preparation I* of *Preparation B* were added 40 ml. of cold acetone and 40 g. of acetone extracted potato starch, kept standing overnight and centrifuged. After that treatment was repeated three times, the supernatant was precipitated with 70 per cent acetone. Enzymes so obtained had appreciable lower maltase and α-limitdextrinase activity per mg. of protein, namely 28 per cent and 33 per cent of *Preparation III* respective-

ly; however there was no detectable trace of amylase activity. As to the question whether maltase and α-limitdextrinase are one enzyme which has dual activity or coexisting different enzymes it is very difficult to decide, because many trials to separate them failed. As a possible mean to approach the answer the author examined whether the maltase which have been recognized to be specific for maltose, acts on α-limitdextrin or not.

The maltase was prepared from the rice-malt following the method of Tokuoka (*21*). The enzyme activity was tested by mixing 1 ml. of the enzyme solution, 3 ml. of 0.1 per cent maltose or α-limitdextrin solution, 2 ml. of acetate buffer at pH 5.0 and 4 ml. of distilled water. The whole mixture was incubated at 40° and the increase of reducing power represented as glucose was determined by the method of Hagedorn and Jensen. The data are summarized in Table V which indicate that the enzyme has an ability to digest the α-limitdextrin and maltose. The so-called maltase may also have the actions of isomaltase and glucosidase.

TABLE V

The Action of Maltase on Maltose and α-Limitdextrin

(The values represent the hydrolysis percentage as glucose.)

Time \ Substrate	Maltose	α-Limitdextrin
hours	*per cent*	*per cent*
1	84	70
3	94	88
5	97	95
6	98	98

SUMMARY

The enzymes which were prepared from the intestinal juice and mucosa of the cattle digested amylose, mltose and α-limitdextrin. The existence of α-amylase action may probably be due to the contamination of pancreatic α-amylase. There was some parallelism between maltase and α-limitdextrinase and the so-called maltase which was prepared from the ricemalt had an ability of digesting α-limitdextrin. The electrophoretical pattern of enzymes showed four peaks.

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